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Title: Chronic Exposure of Rodents to Indole-3-Carbinol and 3,3'-Diindolylmethane: Implications for Drug Metabolism, Chemoprevention and Human Health.

Abstract approved:

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David E. Williams

Indole-3-carbinol (I3C) is a naturally occurring plant alkaloid, found in significant concentrations in cruciferous vegetables such as broccoli and Brussels sprouts. I3C is an unstable compound that undergoes rapid oligomerization in an acidic environment to form higher order condensation products (I3C-ACPs), such as 3-3'-diindolylmethane (DIM). Both I3C and DIM are marketed as dietary supplements and are under investigation as potential chemopreventive agents, despite limited data on the effects of chronic exposure. Previous studies have demonstrated that the chemopreventive potential of I3C and DIM in animal studies is dependent on species, strain, tissue and timing of treatment relative to carcinogen

exposure, and long-term post-initiation exposure can even promote tumors. The majority of biological effects from I3C are the result of the abilities DIM and other I3C-ACPs to bind to the aryl hydrocarbon receptor and the subsequent induction of phase I and phase II enzymes. Phase I and phase II enzyme induction in many cases leads to protection from carcinogens by increasing the rate of metabolism and excretion but in some cases enhances carcinogenicity by increasing the rate of bioactivation. It has been demonstrated that modulation of enzyme levels can also result in altered metabolism of compounds that could affect efficacy and toxicity of pharmaceuticals and xenobiotics. The current work utilizes chronic dietary I3C and DIM exposures in rodent models to further elucidate the effect these compounds might have on health, drug metabolism and carcinogenesis. The reduced weight of Fischer 344 rats treated with 2500 ppm I3C for 1 year may be indicative of adverse effects but toxicity was not confirmed by blood chemistry or histopathological examination. Furthermore, no toxicity was observed after a comparable treatment of Sprague-Dawley rats. As observed after acute and sub-chronic exposures to I3C and DIM, we documented significant induction of cytochrome P450 enzymes and a related modification to drug metabolism in liver slice incubations. Evidence is also provided that may suggest that tumor modulation in mice may occur through an estrogenic mechanism. Further studies should be completed to determine the potential for similar responses in humans.

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Chronic Exposure of Rodents to Indole-3-Carbinol and 3,3'-Diindolylmethane: Implications for Drug Metabolism, Chemoprevention and Human Health

by

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Dustin A. Leibelt, Author

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This thesis is dedicated to my parents,
Boyd and Cindy

CHRONIC EXPOSURE OF RODENTS TO INDOLE-3-CARBINOL AND 3,3'-DIINDOLYLMETHANE: IMPLICATIONS FOR DRUG METABOLISM, CHEMOPREVENTION AND HUMAN HEALTH

CHAPTER 1

INTRODUCTION

In 2002, 1.3 million new cases of cancer, along with 555,500 cancer deaths were expected in the United States, and despite breakthroughs in early detection and cancer treatment, the latest studies found that the 5 year survival rate for all cancers was only 62% (American Cancer Society, 2002). Statistics such as these have driven research in cancer prevention over the last two decades.

In a landmark paper by Doll and Peto (1981) the first evidence suggesting a strong association between dietary components and cancer risk was presented. These epidemiology data, along with supporting animal studies (Rogers *et al.*, 1993), lead health authorities to make dietary recommendations to decrease cancer risk (Bal and Foerster, 1991; Dwyer, 1993). In subsequent studies, epidemiological data linked diets high in fruits and vegetables with a decrease in cancer incidence (Block *et al.*, 1992; Steinmetz and Potter, 1996) and stimulated further research into the area of phytochemicals and their ability to provide chemoprotection against cancer (Greenwald and McDonald, 1997; Greenwald, 1999; and Kelloff *et. al.*,

2000). This research has lead to the identification of numerous possible chemopreventive agents, one of the most promising being indole-3-carbinol (I3C).

I3C is a naturally occurring plant alkaloid formed from the hydrolysis of indole glucosinolate (glucobrassicin), found in significant concentrations in cruciferous vegetables such as broccoli, cauliflower, and Brussels sprouts (Fenwick *et al.*, 1983; Slominski and Campbell, 1987; McDannell *et al.*, 1988; Preobrazhenskaya *et al.*, 1993). Glucobrassicin is hydrolyzed to glucose, sulfate, thiocyanate and I3C, via the intermediate 3-indolylmethyl isothiocyanate, upon maceration or cutting of plant tissue at neutral pH, in the presence of the enzyme myrosinase (Figure 1.1).

I3C is a relatively unstable compound and can condense with itself to form 3,3'-diindolylmethane (DIM) or combine with L-ascorbic acid, which is also found at high levels in cruciferous plants, to form ascorbigen (Figure 1.1). In an acidic environment, such as the stomach after oral exposure, I3C undergoes rapid oligomerization to form dimers, trimers, tetramers, and several other higher order condensation products (I3C-ACP) (Figure 1.2) (Leete and Marion, 1953; Bradfield and Bjeldanes, 1987; Bjeldanes *et al.*, 1991; De Kruif *et al.*, 1991; Grose and Bjeldanes, 1992; Wortelboer *et al.*, 1992). A number of these compounds have been identified including 3,3'-diindolylmethane (DIM), 2-(indol-3-ylmethyl)-3,3'-

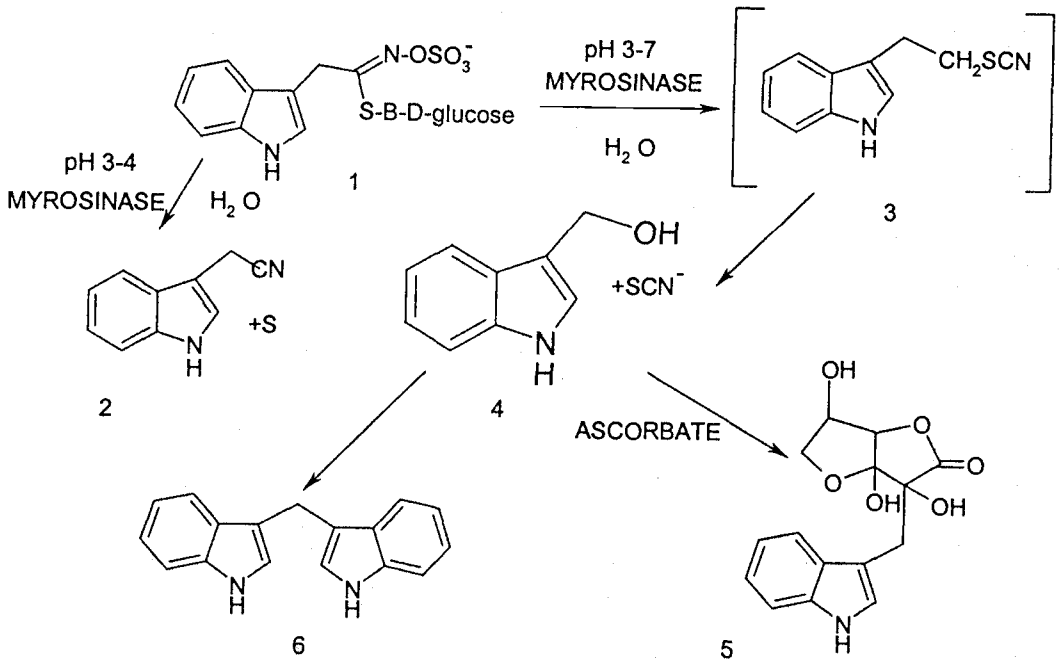

Figure 1.1: Enzymatic hydrolysis of glucobrassicin, found in cruciferous vegetables, and formation of I3C. 1 = Glucobrassicin; 2 = Indole-3-acetonitrile; 3 = 3-Indolylmethyl isothiocyanate; 4 = Indole-3-carbinol(I3C); 5 = Ascorbigen; 6 = 3,3'- Diindolylmethane (DIM) (from McDanell *et al.*, 1988).

diindolylmethane (linear trimer or LT_1), 5,6,11,12,17,18-hexahydrocyclonona[1,2-b:4,5-b':7,8-b"]triindole (cyclic trimer or CT) and indolo[3,2-b]carbazole (ICZ). The major product formed *in vitro* (Spande, 1979) and *in vivo* after oral administration (Dashwood *et al.*, 1989; Stresser *et al.*, 1995a) is the dimer, DIM.

Most of what will be discussed in this thesis is in reference to the parent compound I3C, but it must be kept in mind that I3C is inactive until formation of

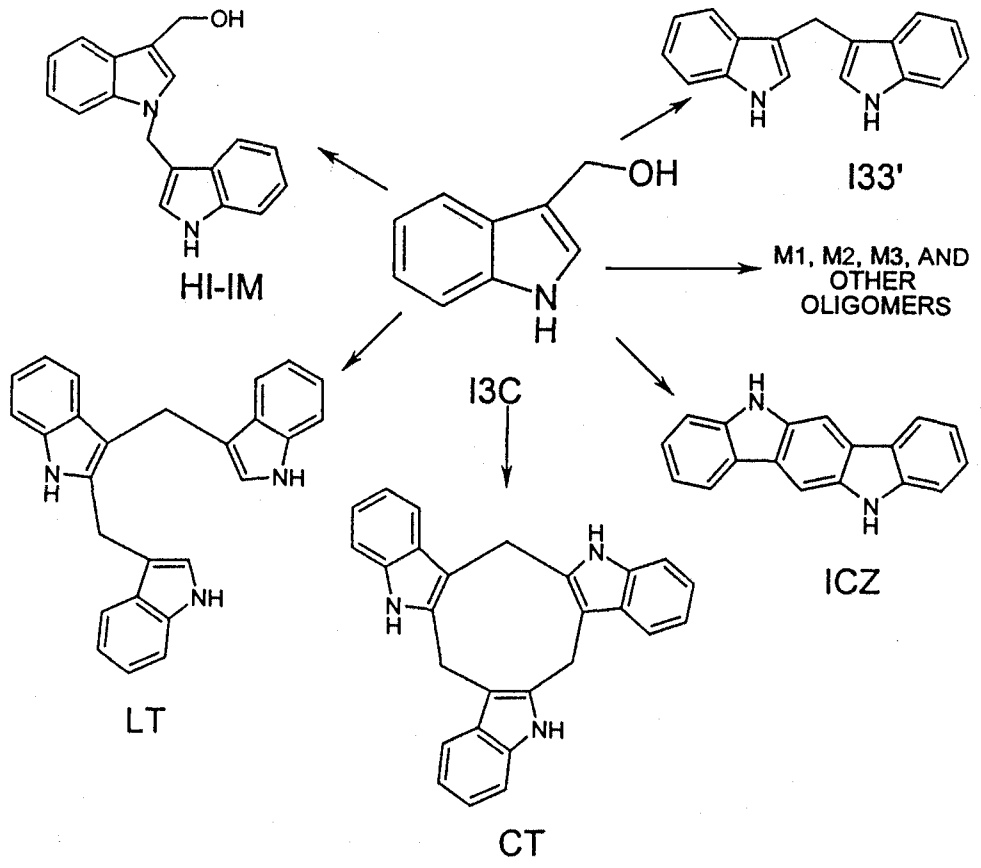

Figure 1.2: Structure of I3C acid condensation products found in liver extracts of rats given I3C orally. I33', 3,3'-diindolylmethane; LT, [2-(indol-3-ylmethyl)-indol-3-yl]indol-3-ylmethane; HI-IM, (hydroxymethyl)indolyl-3-indolylmethane; CT, 5,6,11,12,17,18-hexahydroclonal[1,2-b:4,5-b':7,8-b]:triindole; ICZ, 3,2-b-indolocarbazole. I3C itself was not detected in liver extracts. (Taken from Stresser *et al.*, 1995).

the above condensation products occurs. When administered iv and the stomach is bypassed, the biological efficacy of I3C is eliminated. The explanation for this is that the majority of I3C-ACP bind with relatively high affinity to the aryl hydrocarbon receptor (AHR) when compared to the parent compound (K_ds of 90, 20, 60 and 0.19 nM for DIM, LT_1, CT, and ICZ, respectively compared to 0.007

nM for TCDD and 27,000 nM for I3C) (Bjeldanes *et al.,* 1991). The hypothesis that the majority of effects elicited by I3C are actually actions of I3C-ACP through the AHR is relevant in that the levels of these derivatives in rat and trout liver tissue after administration of ^{3}H-I3C were found to be in the μM range (Dashwood *et al.,* 1989; Stresser *et al.,* 1995a).

I3C and DIM are now both made synthetically and marketed as dietary supplements. I3C is also under evaluation for chemoprotection of women against breast cancer (Wong *et al.,* 1997; Lawrence *et al.,* 2000) and cervical dysplasia (Bell *et al.*, 2000). I3C has been in trials (Rosen *et al.*, 1998) and both I3C and DIM are utilized as treatments for recurrent respiratory papillomatosis (RRP). Besides chemoprotection, their proposed health benefits include promoting beneficial estrogen metabolism (Zeligs, 1999), providing antioxidant protection, boosting the immune system, and acting as a phytochemical replacement for those who lack cruciferous vegetables in their diet. I3C and DIM are rapidly becoming two of the top selling phytochemical supplements.

I3C and DIM in chemoprevention

By far the most researched area dealing with I3C and DIM is in chemoprevention. In 1977 it was shown that oral administration of I3C and other indoles prior to and/or with polycyclic aromatic hydrocarbons (PAHs) was chemoprotective (Wattenberg, 1977). Since then, I3C and DIM have been shown

to be chemoprotective in a number of animal models across a variety of target organs (Wattenberg and Loub, 1978; Morse *et al.*, 1990; Bailey *et al.,* 1991; Kojima *et al.*, 1994; Grubbs *et al.*, 1995; Guo *et al.*, 1995; Oganesian *et al.*, 1997; Chen *et al.*, 1998; Manson *et al.*, 1998; Srivastava and Shukla, 1998; Jin *et al.*, 1999). I3C has also been shown to be effective against a wide variety of environmentally relevant chemical carcinogens including nitrosamines (Shertzer, 1984; Fong et al., 1988; Morse et al., 1988), PAHs (Shertzer, 1983; Jongen *et a*l., 1989) and mycotoxins like aflatoxin B_1 (AFB_1) (Nixon *et al.*, 1984).

It has been proposed that the chemoprotection from I3C is most likely due to a blocking mechanism. A blocking agent is a compound that inhibits carcinogenesis by preventing the carcinogen from reaching or reacting with critical target sites. The possible mechanisms behind this blocking action include (i) induction of biotransformation enzymes that deactivate or enhance secretion of carcinogens, (ii) inhibition of enzymes that bioactivate procarcinogens, and (iii) physico-chemico interaction with carcinogen as in nucleophilic trapping of electrophiles or complexing. There are examples in the literature of I3C functioning by all three mechanisms.

Through I3C-ACP interactions with the AHR, I3C has been shown to act as a blocking agent in animal models of cancer chemoprevention (Wortelboer *et al.*, 1992; Guo *et al.*, 1995). The proposed mechanism of action involves induction of phase I and phase II metabolizing enzymes that result in deactivation or increased

excretion of carcinogens (Bjeldanes *et al.*, 1991; Stresser *et al.*, 1994a, 1994b). An example of this is the shift that occurs in AFB_1 metabolism after I3C exposure. The induction of CYP1A in trout and rat by I3C directs metabolism away from the CYP2C11 and CYP3A mediated pathway of AFB_1-8,9-epoxide production, which is known to form adducts with DNA, and toward the formation of AFM_1, which has reduced carcinogenic potency (Cullen *et al.*, 1987; Stresser *et al.*, 1994a; Takahashi *et al.*,1995). The phase II enzymes induced by I3C include UDP-glucuronosyl transferase (UDPGT), glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase (NQO) (Shertzer and Sainsbury, 1991a). The phase II enzymes act by conjugating endogenous hydrophilic groups to xenobiotics leading to diminished activity of activated procarcingens and increased excretion. As an example, I3C induces the Y_c2 isoform of glutathione S-transferase (GST) in the rat, which has been shown to have a high activity towards AFB_1-8,9-exo-epoxide, the ultimate carcinogenic metabolite of AFB_1 (Stresser *et al.*, 1994b; Hayes *et al.*, 1998).

An alternative blocking mechanism, involving inhibition of carcinogen bioactivation, has been proposed based on data indicating that I3C can inhibit DMBA inducedmammary tumorigenesis when given only four hours prior to the carcinogen (Wattenberg, 1990). This proposal was supported by evidence that a number of I3C-ACPs, including DIM, can inhibit the catalytic activity of CYP (Stresser *et al.*, 1995b). The K_i of DIM inhibition of rat and human CYP 1A is 2-8 μM which is comparable to the concentration found in rat liver (3-6 μM) and

human blood (0.4 –1.6 μM) after oral dosing (Stresser *et al.*, 1995a; Arneson *et al.*, 1999). A decrease in CYP activity and the subsequent decrease in bioactivation of carcinogens also appears to be the primary mechanism of I3C protection in the trout model (Fong *et al.*, 1990; Bailey *et al.*, 1992).

A third blocking mechanism involves physico-chemical interaction with the carcinogen. In many cases carcinogens are activated to electrophilic intermediates that can directly bind to macromolecules like DNA. I3C and other indole compounds have been shown to block damage to macromolecules through antioxidant and radical scavenging properties (Shertzer *et al.*, 1988; Wallin *et al.*, 1993) and contribute to chemoprovention as in the protection against benzo[a]pyrene-induced skin tumors in mice (Shertzer *et al.*, 1994).

In other cases the effects that enzyme induction by I3C have on chemoprevention are indirect, as in the metabolism of estradiol by CYP's in the 1 family. CYP1A1 and 1A2 catalyze the 2-hydroxylation of β-estradiol (E_2), whereas CYP1B1 is an effective E_2-4-hydroxylase (Hayes *et al.*, 1996). While it appears that both catechol estrogens are potentially toxic, evidence suggests that 4-OH-E_2 is the more reactive and toxic metabolite (Newbold and Liehr, 2000), and is less likely to be detoxified through methylation by catechol O-methyltransferase (COMT) (Roy *et al.*, 1990). The shift towards higher levels of 2-hydroxy-E_2 that would occur with induced CYP1A1 levels may explain the chemoprotective effect exhibited by I3C or DIM against estrogen dependent cancers (Bradlow *et al.*, 1991;

Jellinck *et al.*, 1993; Telang *et al.*, 1997; Wong *et al.*, 1997; Michnovicz, 1998). Clinical trials with I3C have documented reductions in urinary E_2 levels in both men and women concurrent with an increase in the 2-OH-E_2/16α-OH-E_2 ratio (Michnovicz *et al.*, 1997) and absorption-enhanced DIM also increased this ratio in a pilot clinical study (Zeligs *et al.*, 2002).

Besides direct and indirect actions through the AHR already mentioned, additional mechanisms have been postulated for chemoprevention by I3C, DIM, and other I3C-ACP. I3C and/or I3C-ACP have been shown to induce a G1 cell cycle arrest by down-regulating the expression of cyclin-dependent kinase-6 (Cover *et al.*, 1998). In other studies I3C and I3C-ACP have increased apoptosis in human cancer cells (Ge *et al.*, 1996), inhibited *in vivo* P-glycoprotein-dependent multidrug resistance (Christensen and LeBlanc, 1996), and inhibited invasion and migration of breast tumor cells (Meng *et al.*, 2000). It has also been demonstrated that a specific I3C-ACP, LT_1, inhibits proliferation of both estrogen-responsive and estrogen-independent human breast cancer cell lines and functions both as a weak AHR agonist and an estrogen receptor (ER) antagonist (Chang et al., 1999).

I3C and DIM in Tumor Promotion and Carcinogenesis

Despite the large number of examples where I3C and DIM play a role in chemoprevention and the lack of data suggesting I3C is a complete carcinogen (Dashwood *et al.*, 1991; Oganesian *et al.*, 1999; Stoner *et al.*, 2002), there is some

evidence indicating exposure to I3C can increase cancer risk. Although induction of enzymes make I3C effective as a blocking agent against some carcinogens, in some cases enzyme induction is a cause for concern. Certain CYP isozymes, especially those in the 1A subfamily, can enhance carcinogenicity by increasing the rate of bioactiviation (Ioannides and Parke, 1993); for example the activation of polycyclic aromatic hydrocarbons such as benzo[a]pyrene and aromatic amines such as 4-aminobiphenyl or PhIP. Dietary exposure to I3C elevated tumor incidence in rats initiated with the colon-specific carcinogen 1,2-dimethylhydrazine (1,2-DMH) apparently by enhancing carcinogen binding to DNA (Autrup *et al.*, 1980; Pence *et al.*, 1986). DNA adduct levels were also elevated in the liver of rats exposed to dietary I3C prior to intubation with the tobacco-specific nitrosamine 4-(methylnitroamino)-1-(3-pyridyl)-1-butanone (NNK) (Morse *et al.*, 1988). I3C exposure resulted in a significant increase in liver foci in rats initiated with aflatoxin B_1 (Stoner *et al.*, 2002) and was found to promote hepatocarcinogenesis in trout (Dashwood *et al.*, 1991; Oganesian *et al.*, 1999). The potential for enhancement of tumorigenesis was also demonstrated in a study in which I3C elevated 12-O-tetradecanoyl phorbol-13-acetate induction of ornithine decarboxylase activity in mouse epidermis, signifying tumor promoting activity (Birt *et al.*, 1986). Some studies have even demonstrated that in the presence of nitrites and acid, I3C and its ACPs can be converted to nitrosamines (Tiedink *et al.*,

1989; Sasagawa and Matsushima, 1991). This suggests that under conditions found in the stomach, I3C may indirectly contribute to cancer initiation.

In addition to the previously mentioned antiestrogenic effects that seem to contribute to chemoprevention, I3C and DIM have also been shown to exhibit estrogenic activity in rainbow trout (Shilling and Williams, 2000; Shilling *et al.*, 2001). The estrogenicity of I3C is a likely mechanism by which I3C promotes hepatocarcinogenesis in trout (Oganesian *et al.*, 1999). DIM is primarily an anti-estrogen in mammals (Chen *et al.*, 1998; McDougal *et al.*, 2001) and it has been proposed that this difference may be a function of species-specific DIM metabolism. Preliminary evidence indicates that CYP-dependent hydroxylation of DIM is required in trout to elicit estrogenicity (Shilling *et al.*, 2001).

Toxicity Studies

I3C does not exhibit obvious cytotoxicity (Babich *et al.*, 1993) or mutagenicity (Reddy *et al.*, 1983; Birt *et al.*, 1986; Kuo *et al.*, 1992) *in vitro*, and demonstrates relatively low acute toxicity and limited teratogenic effects *in vivo* when administered orally (Nishie and Daxenbichler, 1980; Dashwood *et al.*, 1991; Shertzer and Sainsbury, 1991a). The LD_{50} in rat after oral dosing was estimated to be more than 2,250 mg/kg body weight in a study sponsored by the Chemoprevention Branch of the National Cancer Institute (NCI).

Conversely, in other NCI sponsored studies, several adverse effects of chronic I3C exposure were revealed. Twenty-eight and 90-day feeding studies were performed in rats and dogs. Doses ranging from 20 to 2000 mg/kg body weight in rats resulted in toxic effects to the hematopoietic system, liver, hair coat and testes, and doses of 20 and 100 mg/kg body weight resulted in an increase in liver weight associated with induction of smooth endoplasmic reticulum. In dogs, diarrhea was observed in the 28- and 90-day studies after administration of I3C at 15 to 150 mg/kg body weight. The 150 mg/kg body weight dose also resulted in diminished body weights, thymic atrophy, anemia and testicular degeneration (Kelloff *et al.*, 1996).

I3C and DIM Effects on Xenobiotic Metabolism

One area of concern for chronic exposure to such phytochemicals is the well documented occurrence of drug-drug interactions as a result of phytochemical induced modulation of metabolizing enzymes. The most prominent example involves alteration of blood levels of drugs in patients consuming grapefruit juice (Bailey *et al.*, 1994). In this case furanocoumarins in the grapefruit juice inhibit CYP3A4, a CYP isoform responsible for the metabolism of 50-60% of all therapeutic drugs (Guengrich, 1999). This results in an improved bioavailability of drugs such as midazolam, cyclosporine, and felodipine leading to an increase in

efficacy or enhanced toxicity depending on the pharmacological properties of the metabolites and the therapeutic index.

The concerns over drug-drug or food-drug interactions via I3C and DIM exposure are substantiated by data from previous studies demonstrating similar enzyme interactions. As previously mentioned, induction of numerous CYP isoforms resulting from dietary exposure of I3C and DIM in animals has been well documented. Short term I3C exposure has produced 2-4 fold inductions of hepatic CYP1A2, CYP2B1/2, and CYP3A with over 20-fold increases observed in CYP1A1 (Stresser *et al.*, 1994a). Chronic exposures, as studied in the current work (Ch 2 & 3), have resulted in even stronger 40- and 30- fold inductions for CYP1A1 and CYP3A2, respectively, depending on dose and strain of rat. Sub-chronic exposures to I3C have also been shown to inhibit flavin-containing monooxygenase (FMO) isoform 1 expression and activity in the rat liver and intestine in a time- and dose-dependent fashion (Larsen-Su and Williams, 1996). For drugs metabolically inactivated by FMO a trend of increasing efficacy or toxicity could occur with increasing exposure to I3C or DIM, whereas the opposite could occur with CYP induction as an increase in metabolism could lead to diminished potency.

The potential effects that the combined CYP induction and FMO inhibition have on drug metabolism were documented by Katchamart *et al.* (2000). This study demonstrated that the ratio of FMO/CYP mediated metabolism of N,N-dimethylaniline, nicotine, and tamoxifen in incubations with liver microsomes

decreased after rats were given 2500 ppm DIM in their diets for 4 weeks. This decrease in metabolite ratio was attributed to an inhibition of FMO-dependent formation of the N-oxide of these tertiary amines with a concurrent induction of CYP-mediated N-demethylation. The concurrent inductions of CYP and inhibition of FMO raises concerns as to the effects I3C and DIM may have on the metabolism of other drugs and xenobiotics.

Other Biological Effects

There is some evidence that I3C and DIM may have effects on the level and activity of other proteins involved in essential metabolic functions. Dunn and LeBlanc (1994) demonstrated that some acid condensation products of I3C lower serum LDL/VLDL cholesterol levels in mice by inhibiting acyl-CoA:cholesterol acyltransferase (ACAT). UDP-glucose dehydrogenase, a cytosolic enzyme responsible for the formation of UDP-glucuronic acid, and NADPH-cytochrome c-reductase and cytochrome b5, proteins involved in microsomal electron transport and xenobiotic metabolism, were found to be elevated in rats exposed to 4100 ppm dietary I3C for 10 days (Cha et al., 1985). A decrease in activity of superoxide dismutase and glutathione peroxidase was observed in rats that received 50 mg/kg I3C for 10 days by gavage (Shertzer and Sainsbury, 1991b). Long-term feeding of 0.5% I3C to rats was shown to inhibit ornithine decarboxylase (ODC) activity

(Manson *et al.*, 1998), the enzyme responsible for the rate limiting step in polyamine synthesis, which is required for DNA replication and cell proliferation.

I3C and DIM Exposure Comparisons

It is very important to consider I3C and DIM dose when making comparisons or extrapolations from observed data to real life situations. The chronic doses of I3C and DIM utilized in the current studies were selected to provide data that would represent the current supplemental doses and not dietary exposures. The recommended maximum human doses of I3C and DIM are 10 mg/kg (Life Extensions™) and 2 mg/kg (BioResponse, LLC), respectively. In comparison, a 300 g serving of Brussels sprouts would provide between .29 and 2.1 mg/kg/day I3C.

Summary

Despite the increasing use of I3C and DIM as dietary supplements, and as treatments for breast cancer, recurrent respiratory papillomatosis (RRP), and cervical intraepithelial neoplasia (CIN), little is known about the effects of chronic dietary exposure. The chemopreventive properties of the compounds have been well documented and acute and sub-chronic studies have failed to reveal significant toxicities. On the other hand, studies demonstrating the promotion of cancer, the lack of long-term exposure data and the observed alterations in CYP levels and

drug metabolism raise concerns relative to chronic exposure of humans to these compounds.

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CHAPTER 2

Effects of Chronic Dietary Indole-3-Carbinol (I3C) Exposure On Blood Chemistry, Histopathology, and Cytochrome P450 in Male Fischer 344 Rats

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Abstract

Indole-3-carbinol (I3C) is a naturally occurring component of the human diet and is found in high concentrations in cruciferous vegetables. I3C is also marketed as a dietary supplement and has been proposed as a chemopreventive agent for women against breast cancer. Acute and sub-chronic exposures of I3C have failed to uncover significant toxicities in the rat but alterations in phase I and phase II drug metabolizing enzymes have been observed. Long-term studies have focused on the effects of chronic I3C exposure on both chemoprevention and tumor promotion. Less is known about the effects of long-term I3C exposure on health and drug/xenobiotic metabolism. Fischer 344 rats were fed diets containing 0, 1000, or 2500 ppm I3C for either 6 or 12 months. Gross pathology of the entire animal along with histopathological examination of the liver, kidneys, adrenals, colon, prostate, and testes yielded no significant lesions in I3C treated rats. A small animal chemistry panel was performed and serum testosterone levels determined. The results of the chemistry panel did not indicate any level of toxicity, and in fact there was a general trend of decreasing serum enzyme levels (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, creatine kinase) with increasing I3C dose after 6 and 12 months. Testosterone levels were slightly reduced in the rat serum after 6 months, but significantly elevated after 12 months of exposure. The only other significant findings in the serum analysis were an increase in globulin levels after 6 months, an increase in amylase levels after 12

months and a decrease in calcium levels also after 12 months. The total cytochrome P450 (CYP) of the liver was determined along with the levels of the specific isoforms CYP1A1/2 and CYP3A2. As seen in sub-chronic studies, the liver total CYP content was significantly induced, as were CYP1A1/2 and CYP3A2 levels. While confirming that I3C is relatively non-toxic in the rat, the alterations observed in this study following long-term dietary I3C administration raise concerns relative to potential adverse effects on hormone levels and drug/xenobiotic metabolism.

Introduction

I3C is a naturally occurring plant alkaloid formed from the hydrolysis of indole glucosinolate (glucobrassicin), found in significant concentrations in cruciferous vegetables such as broccoli, cauliflower, and Brussels sprouts (Slominski and Campbell, 1987; McDannell *et al.*, 1988). Glucobrassicin is hydrolyzed to glucose, sulfate, thiocyanate and I3C upon maceration of plant tissue at neutral pH, in the presence of the enzyme myrosinase. Even at neutral pH, I3C is relatively unstable and readily condenses with itself to form 3,3'-diindolylmethane (DIM) or combines with L-ascorbic acid, which is also found at high levels in cruciferous plants, to form ascorbigen. In an acidic environment, such as the stomach after oral exposure, I3C undergoes rapid oligomerization to form dimers, trimers, tetramers, and several other higher order acid condensation products (I3C-ACP) (Bjeldanes *et al.*, 1991; De Kruif *et al.*, 1991; Grose and Bjeldanes, 1992; Wortelboer *et al.*, 1992). A number of these compounds have been identified including 3,3'-diindolylmethane (DIM), 2-(indol-3-ylmethyl)-3,3'-diindolylmethane (linear trimer or LT_1), 5,6,11,12,17,18-hexahydrocyclonona[1,2-b:4,5-b':7,8-b"]triindole (cyclic trimer or CT) and indolo[3,2-b]carbazole (ICZ).

I3C itself does not become biologically/pharmacologically active until the above acid condensation products are formed. In fact, when I3C is administered iv and the stomach is bypassed, biological efficacy is eliminated. The explanation for this is that the majority of I3C-ACPs bind with relatively high affinity to the aryl

hydrocarbon receptor (AHR) when compared to the parent compound (K_ds of 90, 20, 60 and 0.19 nM for DIM, LT_1, CT, and ICZ, respectively compared to 0.007 nM for TCDD and 27,000 nM for I3C) (Bjeldanes *et al.,* 1991). The hypothesis that the majority of effects elicited by I3C are actually actions of I3C-ACP through the AHR is relevant in that the levels of these derivatives in rat and trout liver tissue after administration of ^{3}H-I3C were found to be in the μM range (Dashwood *et al.,* 1989; Stresser *et al.,* 1995).

Through I3C-ACP interactions with the AHR, I3C has been shown to act as a blocking agent in animal models of cancer chemoprevention (Wortelboer *et al.*, 1992; Guo *et al.,*1995). The proposed mechanism of action involves AHR-dependent induction of phase I and phase II enzymes (Bjeldanes *et al.,* 1991; Stresser *et al.,* 1994). The induction of phase I enzymes such as CYP1A1/2, CYP2B1/2, and CYP3A1/2 in many cases leads to protection from carcinogens by increasing their rate of oxidative metabolism to less toxic metabolites that are more easily excreted. An example of this is the detoxification of aflatoxin B_1 (AFB_1) to aflatoxin M_1 (AFM_1) and aflatoxin Q_1 (AFQ_1) by CYP1A and CYP3A, respectively (Stresser *et al.*, 1994). Phase II enzymes induced include UDP-glucuronosyl transferase, glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase (Shertzer and Sainsbury, 1991). These phase II enzymes act by adding endogenous hydrophilic groups to xenobiotics leading to diminished activity of activated procarcinogens and increased excretion.

In some instances, induction of enzymes can be a cause for concern. Certain CYP isozymes, especially in the 1A subfamily, can enhance carcinogenicity by increasing the rate of bioactivation (Ioannides and Parke, 1993); for example the activation of polycyclic aromatic hydrocarbons such as benzo[a]pyrene and aromatic amines such as 4-aminobiphenyl or PhIP.

In other cases the effects that enzyme induction has on carcinogenesis are indirect, as in the metabolism of estradiol by CYPs in the 1 family. CYP1A1 and 1A2 catalyze the 2-hydroxylation of β-estradiol (E_2), whereas CYP1B1 is an effective E_2-4-hydroxylase (Hayes *et al.*, 1996). While it appears that both catechol estrogens are potentially toxic, evidence suggests that 4-OH-E_2 is the more reactive and toxic metabolite (Newbold and Liehr, 2000), and is less likely to be detoxified through methylation by catechol O-methyltransferase (COMT) (Roy *et al.*, 1990). The shift towards higher levels of 2-hydroxy-E_2 that would occur with induced CYP1A1 levels may explain the chemoprotective effect exhibited by I3C or DIM against estrogen dependent cancers (Bradlow *et al.*, 1991; Jellinck *et al.*, 1993; Telang *et al.*, 1997; Wong *et al.*, 1997; Michnovicz *et al.*, 1998).

Although effective as a blocking agent, long-term post-initiation exposure to I3C, as would most likely occur in humans, has shown the potential to promote hepatocarcinogenesis. Chronic I3C exposure resulted in a significant increase in liver foci in rats initiated with aflatoxin B_1 (Stoner *et al.*, 2002) and was found to

promote hepatocarcinogenesis in trout (Dashwood *et al.*, 1991; Oganesian *et al.*, 1999).

The health consequences of CYP enzyme induction are not limited to carcinogen bioactivation/detoxification or tumor promotion. Phase I enzymes such as CYP 3A make up approximately 40% of the total hepatic CYP content in human liver and play an important role in drug metabolism as 50-60% of all clinically relevant drugs are metabolized by this subfamily (Guengrich, 1999). As an example of a potential I3C-drug interaction, studies have shown differential metabolism of tamoxifen and nicotine by liver microsomes from rats fed I3C (Katchamart *et al.*, 2000).

Despite the possible promotional properties and the potential effects I3C could have on drug metabolism, little evidence of direct toxicity by I3C has been uncovered. I3C does not exhibit obvious cytotoxicity (Babich *et al.,* 1993) or mutagenicity (Reddy *et al.,* 1983; Birt *et al.*, 1986; Kuo *et al.,* 1992) *in vitro*, and demonstrates relatively low acute toxicity and limited teratogenic effects *in vivo* when administered orally (Nishie and Daxenbichler, 1980; Dashwood *et al.*, 1991; Shertzer and Sainsbury, 1991). The LD_{50} in rats after oral dosing was estimated to be more than 2,250 mg/kg body weight in a study sponsored by the Chemoprevention Branch of the National Cancer Institute (NCI).

Conversely, other NCI sponsored studies have revealed several adverse effects of sub-chronic I3C exposure in rats and dogs. In rats, doses ranging from 20

to 2000 mg/kg body weight resulted in toxic effects to the hematopoietic system, liver, hair coat and testes, and doses of 20 and 100 mg/kg body weight resulted in an increase in liver weight associated with induction of smooth endoplasmic reticulum. A study in which prenatal I3C exposure resulted in adverse reproductive outcomes in male Sprague-Dawley rats is a notable exception to the normal absence of teratogenic effects (Wilker *et al.*, 1996). In dogs, diarrhea was observed in 28- and 90-day studies after administration of 15 to 150 mg/kg body weight. The 150 mg/kg body weight dose of I3C also resulted in diminished body weights, thymic atrophy, anemia and testicular degeneration (Kelloff *et al.*, 1996).

I3C is now marketed as a dietary supplement and has been advocated as a chemoproventive agent for women against breast cancer (Wong *et al.,* 1997; Lawrence *et al.,* 2000) despite the fact that little is known about the effects of long-term exposure. The changes that short-term I3C exposure could produce in drug metabolism and carcinogen activation, along with the previously mentioned toxicities and evidence indicating that I3C can sometimes act as a tumor promoter (Dashwood *et al.,* 1991; Oganesian *et al.* 1999; Stoner *et al.*, 2002), raise concerns as to whether long-term treatment is safe in humans. In this study we use blood chemistry panels and histopathology of relevant tissues in an attempt to reveal any potential toxicities in the rat, while also investigating the effect of chronic I3C exposure on drug and carcinogen metabolizing enzymes.

Materials and Methods

Chemicals

Indole-3-carbinol was obtained from Sigma-Aldrich Co. (Milwaukee, WI).

Animals

Twenty-seven male Fischer 344 rats were purchased from Simonsens (Gilroy, CA) at four weeks of age. Rats were housed individually in hanging metal wire cages at the Laboratory Animal Resource Center, Oregon State University and maintained at 22°C and 40 to 60 % humidity on a 12 hour light/dark cycle. Both tap water and powdered semisynthetic diet (AIN-76A) were available *ad libitum* throughout the study. After a one week acclimation period, animals were randomly divided into 3 different treatment groups, each containing 9 rats (Table 2.1) and I3C was incorporated into the diet at 0, 1000, or 2500 ppm. At the end of 6 months (5 animals per group) or one year (4 animals per group) rats were weighed and all diets were removed overnight before sampling. Rats were anesthetized and blood samples were collected as described in the following section. While remaining under anesthesia, rats were euthanized by exsanguination. Gross pathology was performed and a portion of the liver, colon, adrenal, kidneys, testes and prostate was preserved in neutral buffered formalin for subsequent sectioning and histopathology. A second portion of the liver was removed prior to fixation and immediately frozen in liquid nitrogen and then stored at -80° C until analysis. The

above protocols were approved by the Oregon State University Institutional Animal Care and Use Committee.

TABLE 2.1. STUDY DESIGN

Group	Duration	Supplement	Daily Dose	Rats (N)
1A	6 months	-	None	5
2A	6 months	I3C	1000 ppm	5
3A	6 months	I3C	2500 ppm	5
1B	12 months	-	None	4
2B	12 months	I3C	1000 ppm	4
3B	12 months	I3C	2500 ppm	4

Four week old Fischer 344 rats were obtained commercially, acclimated for 1 week and then randomly assigned to 1 of the 3 treatment groups. The housing, preparation of diet and experimental details are described in Materials and Methods.

Blood collection and analysis

Blood was collected from the pre-cava while rats were anesthetized under 4% isoflurane with 0_2 at a flow of 2 L/min. Blood was stored at 4°C for 2 hours and then spun for 20 minutes at 11,000 rpm to isolate serum. An aliquot of serum was sent to the Texas Veterinary Medical Diagnostic Laboratory (College Station, TX) where testosterone levels were determined by a commercial radioimmunoassay from Diagnostic Products Corporation (Los Angeles, CA) and a small animal

clinical chemistry analysis was performed utilizing a Hitachi 911 Chemical Analyzer (Roche, Indianapolis, IN).

Histopathology

Tissues were fixed in 10% neutral buffered formalin and processed on a LX300 Tissue Processor (Fisher Scientific). Sections were cut at 4-5 microns and stained on the S/P Automatic Slide Stainer GLX with Haematoxylin (Gill-3, Shandon Inc.-Pittsburgh, PA) and Eosin Y (alcoholic, Shandon Inc.-Pittsburgh, PA). Slides were coverslipped with Shur/Mount (Triangle Biomedical Sciences-Durham, NC).

Microsome preparation and total CYP

Liver samples were homogenized with Brinkmann Polytron (Westbury, NY) into 3 volumes of cold homogenization buffer (10 mM potassium phosphate [pH 7.5] containing 0.15 M potassium chloride, 1 mM EDTA, and 0.1 mM phenylmethylsulfonylfluoride (PMSF)). Microsomes were prepared by ultracentrifugation according to Guengerich (1989). Protein levels were determined by the method of Lowry *et al.* (1951). The total liver microsomal CYP content was quantified by the CO versus CO-reduced difference spectra (Omura and Sato, 1964) on a Cary 300 UV-Vis spectrophotometer (Varian, Walnut Creek, CA).

Electrophoresis and immunoblotting

Microsomal proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and

electrophoretically transferred to nitrocellulose membrane (Towbin et al., 1979). The membranes were incubated with goat antibodies recognizing rat CYP1A1/2 (Oxygene[2], Dallas, TX) or CYP3A2 (Gentest, Woburn, MA) and probed with rabbit anti-goat secondary antibody conjugated with horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The blots were visualized by a chemiluminescence detection kit (New England Nuclear, Boston, MA) and densitometry was performed using an HP Scanjet IIcx flatbed scanner and NIH Image software version 1.61/ppc (public domain, National Institutes of Health).

[2]No longer in business.

Statistics

Treatments were compared separately within the 1 year and 6 month data sets. The three treatments (control, I3C-1000 and I3C-2500) were compared using ANOVA followed by pairwise comparisons and linear contrasts whenever data (on original or log transformed scale) were consistent with assumptions of the methods (homogenous normal errors). When apparent outliers were present, non-parametric rank methods were used (Kruskal-Wallis test followed by Wilcon tests for pairwise comparisons). When heterogeneous normal errors were present, a mixed linear model allowing heterogeneous errors by treatment was used (approximate F-tests followed by approximate t-tests both using the Kenward-Rogers adjustment in the Mixed Procedure of SAS). Analyses were conducted using SAS version 8.2 (SAS Institute, Inc., Cary, NC (2001)).

Results

Body weights

At time of sacrifice there was significant evidence of a treatment related reduction in body weight for rats at both 6 ($p=0.015$) and 12 ($p=0.006$) months (Figure 2.1). Differences of 11 and 13 % in weight were observed between rats receiving control diet and 2500 ppm I3C for 6 and 12 months, respectively.

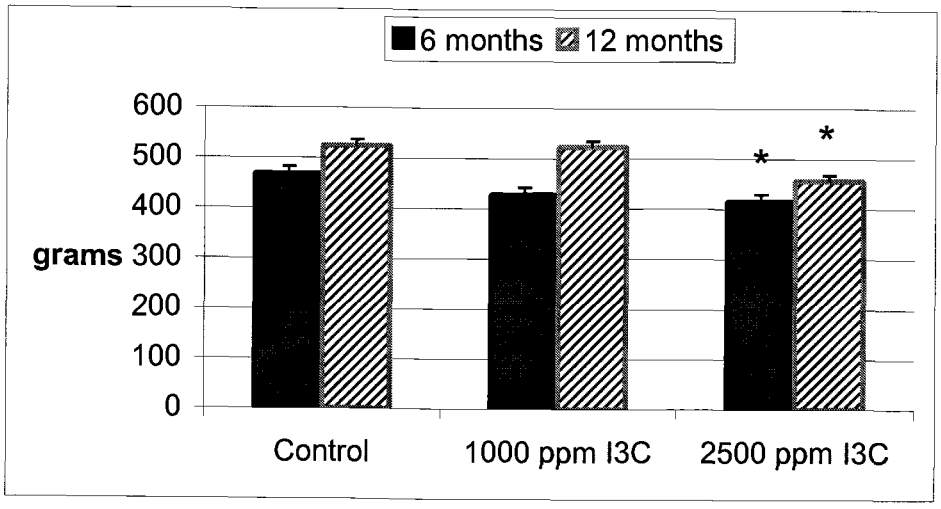

Figure 2.1: Body weights of rats fed control, 1000 ppm I3C or 2500 ppm I3C for 6 or 12 months. * = $p<0.05$ compared to corresponding control group.

Clinical chemistry and testosterone levels

Small animal chemistry panels of rat serum revealed insignificant differences except for the level of globulin after 6 months of exposure (Table 2.2), the levels of calcium and amylase after 12 months (Table 2.3), and a general

TABLE 2.2. CLINICAL CHEMISTRY FOR RATS AFTER 6 MONTHS OF DIETARY EXPOSURE

Group	Serum Protein	Albumin	Calcium	Phosphorous	Glucose	BUN
1A	7.2±.09	4.9±.13	9.9±.08	5.8±.22	165±30	23.2±1.1
2A	7.2±.10	4.7±.14	9.9±.07	5.6±.19	173±7	22.8±0.6
3A	7.3±.14	4.4±.13	10.1±.10	5.2±.28	177±10	21.3±1.8

Group	Creatinine	Bilirubin	Globulin	GGT	Amylase	Cholesterol
1A	0.12±.02	1.8±0	2.3±.09	<3	2946±47	184±17
2A	0.12±.02	1.8±0	2.6±.05*	<3	2726±48	162±8
3A	0.16±.04	1.8±0	2.9±.03*	<3	2831±98	221±9

*** = p< 0.05 compared to corresponding control group.**

TABLE 2.3. CLINICAL CHEMISTRY FOR RATS AFTER 12 MONTHS OF DIETARY EXPOSURE

Group	Serum Protein	Albumin	Calcium	Phosphorous	Glucose	BUN
1B	6.1±0	4.0±.13	10.7±.21	5.5±.40	202±13	38.8±8.2
2B	6.1±0	3.7±.18	10.1±.18*	5.3±.23	184±22	34.7±8.9
3B	6.1±0	3.9±.19	10.1±.09*	5.5±.33	191±9	26.0±5.7

Group	Creatinine	Bilirubin	Globulin	GGT	Amylase	Cholesterol
1B	.38±.10	1.11±.26	2.2±0.1	<3	2683±73	271±29
2B	.33±.08	0.78±.32	2.4±.29	<3	2430±323	243±47
3B	.20±.10	0.37±.03	2.3±.09	<3	3069±140*	210±27

*** = p< 0.05 compared to corresponding control group.**

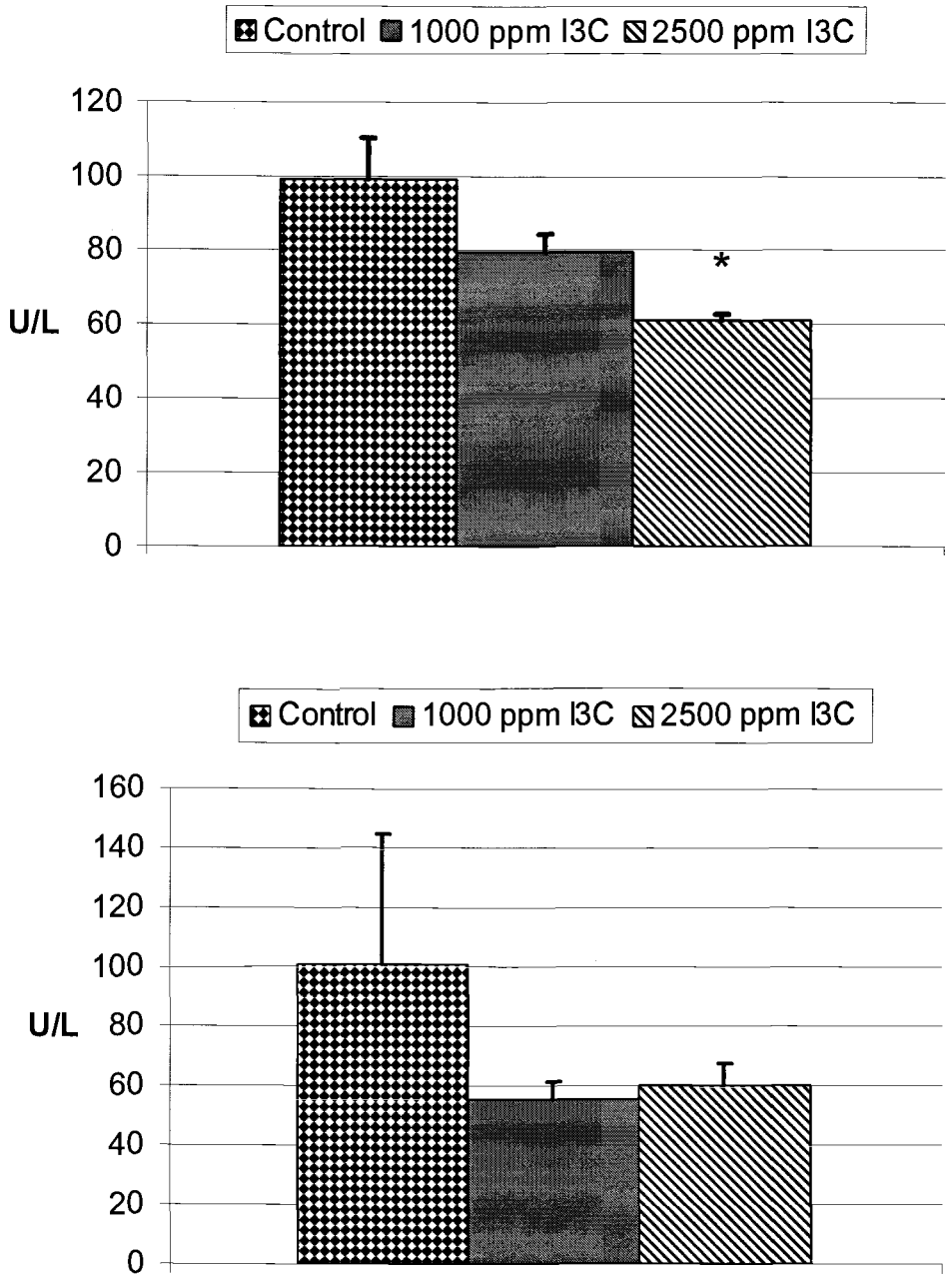

Figure 2.2: Serum aspartate aminotransferase levels in male Fischer 344 rats after 6 (Top) or 12 (bottom) months dietary I3C exposure. Values represent group averages in U/L $\pm$ SE. * = $p<0.05$ compared to corresponding control group.

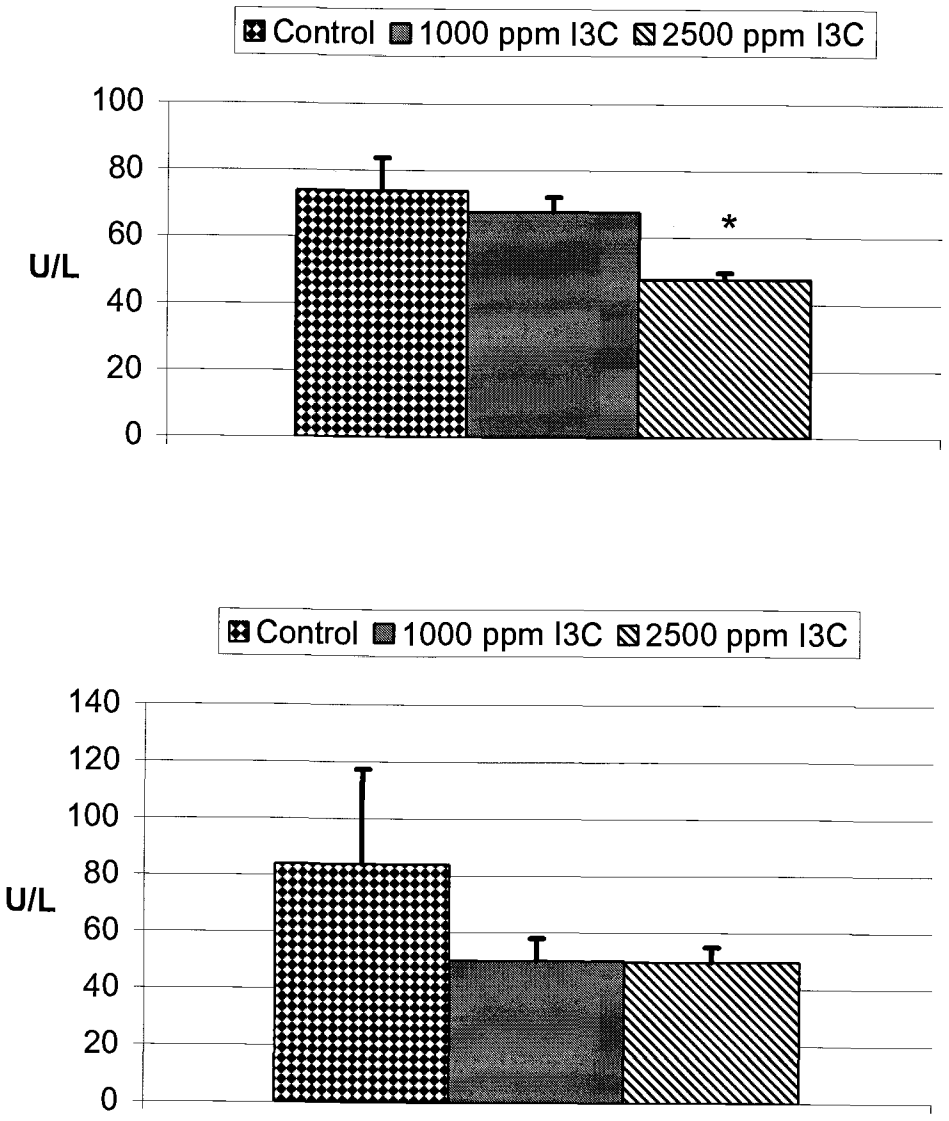

Figure 2.3: Serum alanine aminotransferase levels in male Fischer 344 rats after 6 (Top) or 12 (Bottom) months dietary I3C exposure. Values represent group averages in U/L $\pm$ SE. * = $p<0.05$ compared to corresponding control group.

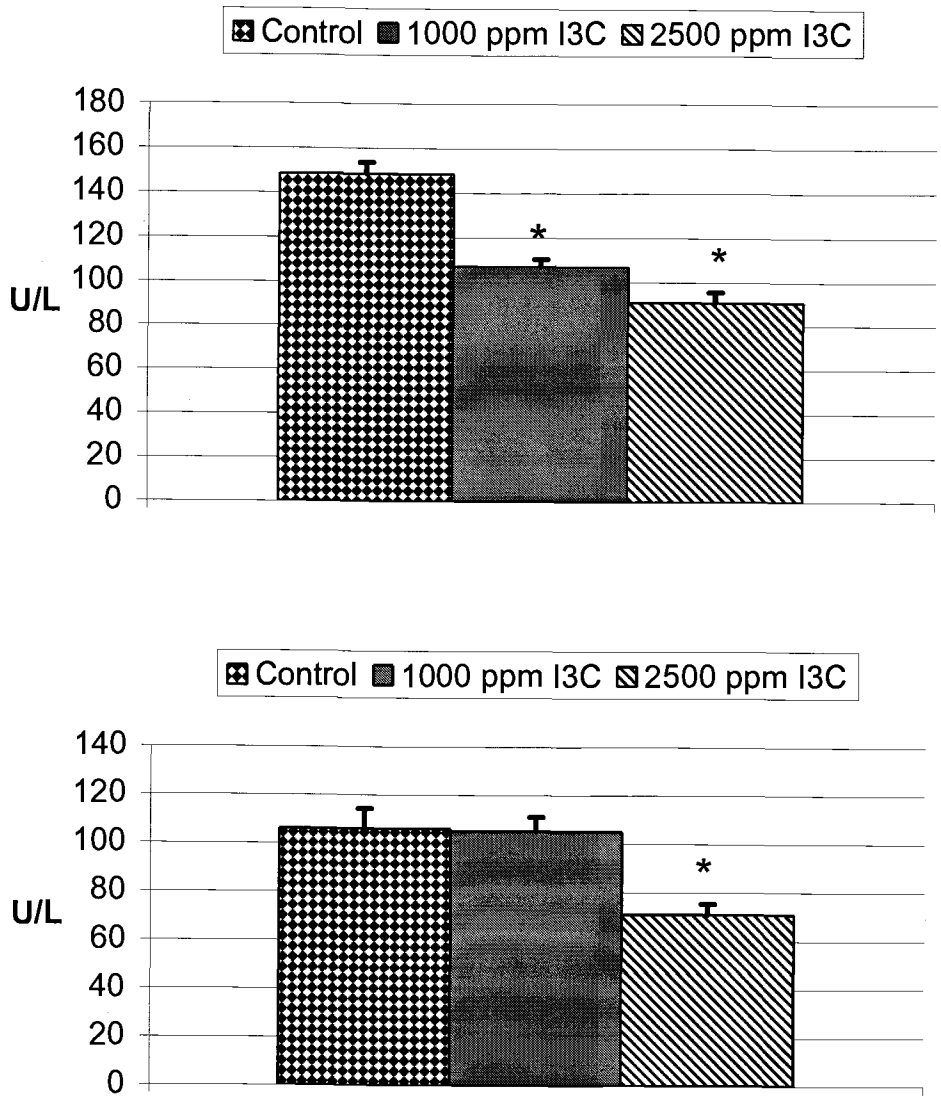

Figure 2.4: Serum alkaline phosphatase levels in male Fischer 344 rats after 6 (Top) or 12 (Bottom) months dietary I3C exposure. Values represent group averages in U/L $\pm$ SE. * = $p<0.05$ compared to corresponding control group.

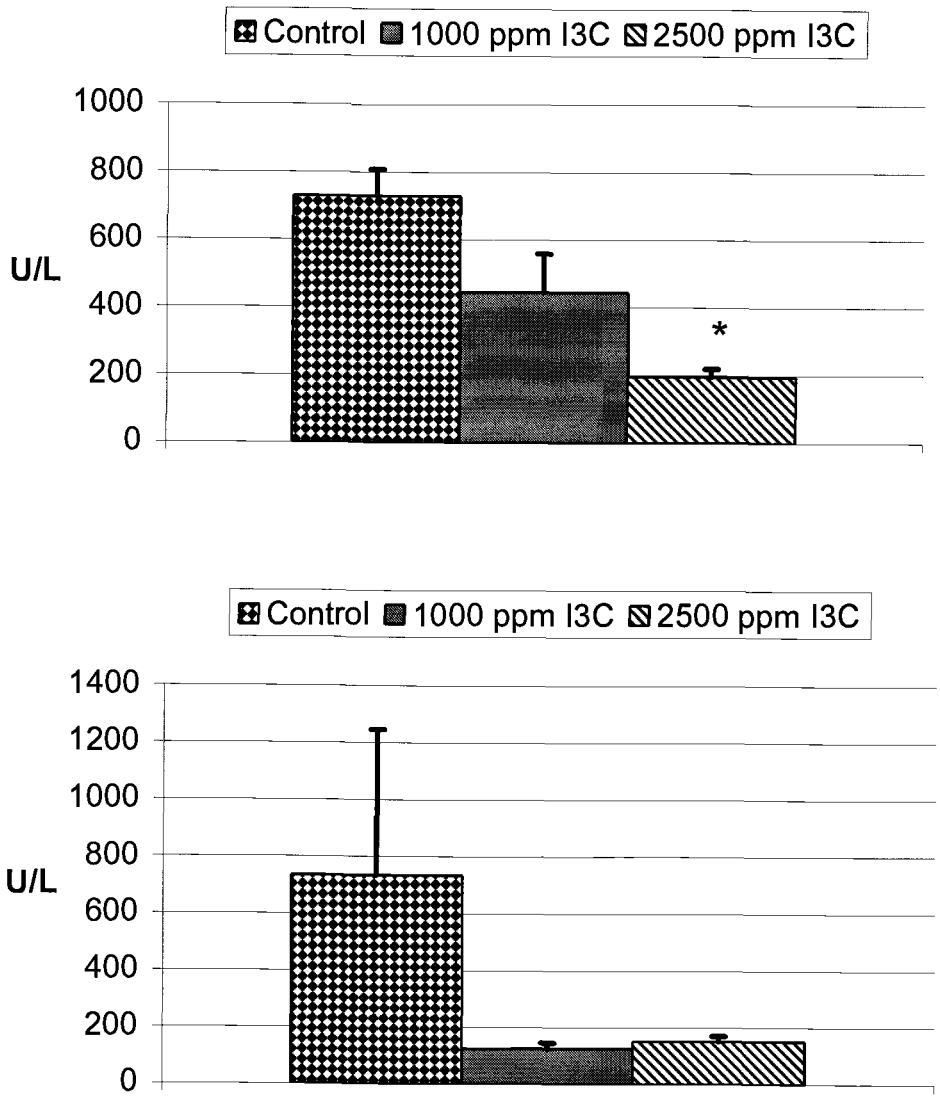

Figure 2.5: Serum creatine kinase levels in male Fischer 344 rats after 6 (Top) or 12 (Bottom) months dietary I3C exposure. Values represent group averages in U/L ± SE. * = $p<0.05$ compared to corresponding control group.

decrease in serum enzyme levels at both time points (Figures 2.2 – 2.5). At six months a strong linear trend of increasing globulins with increasing I3C dose was observed ($p<0.0001$) but the trend was not evident in the 12 month data. After 12 months of exposure, a linear trend of decreasing calcium levels with increasing I3C was observed ($p=0.0149$), along with a significant treatment related effect on amylase levels ($p=0.016$). As mentioned above, there is a trend of decreasing serum enzyme levels with an increase in I3C exposure at both 6 and 12 months. This decrease was significant for alkaline phosphatase ($p<0.0001$), creatine kinase ($p=0.0003$), aspartate aminotransferase ($p=0.0062$), and alanine aminotransferase ($p=0.017$) after 6 months, but for alkaline phosphatase only after 12 months. Analysis of serum testosterone levels lead to opposite results at 6 and 12 months (Figure 2.6). After 12 months there was evidence of an increasing trend with dose ($p=0.038$) and at 6 months an insignificant decreasing trend ($p=0.059$).

Histopathology

No significant differences between groups were noted upon necropsy or following histopathology at 6 or 12 months. Most notably, no toxicities were indicated in treatment groups as compared to controls in the liver (Figure 2.7) and no effects were seen in hormone responsive tissues such as prostate and testes (Figures 2.8 and 2.9). There were numerous large hyaline casts found in the kidney tubules. The appearances of such casts are common in rats with age (Lord and Newberue, 1990), and no treatment related differences were evident (Figure 2.10).

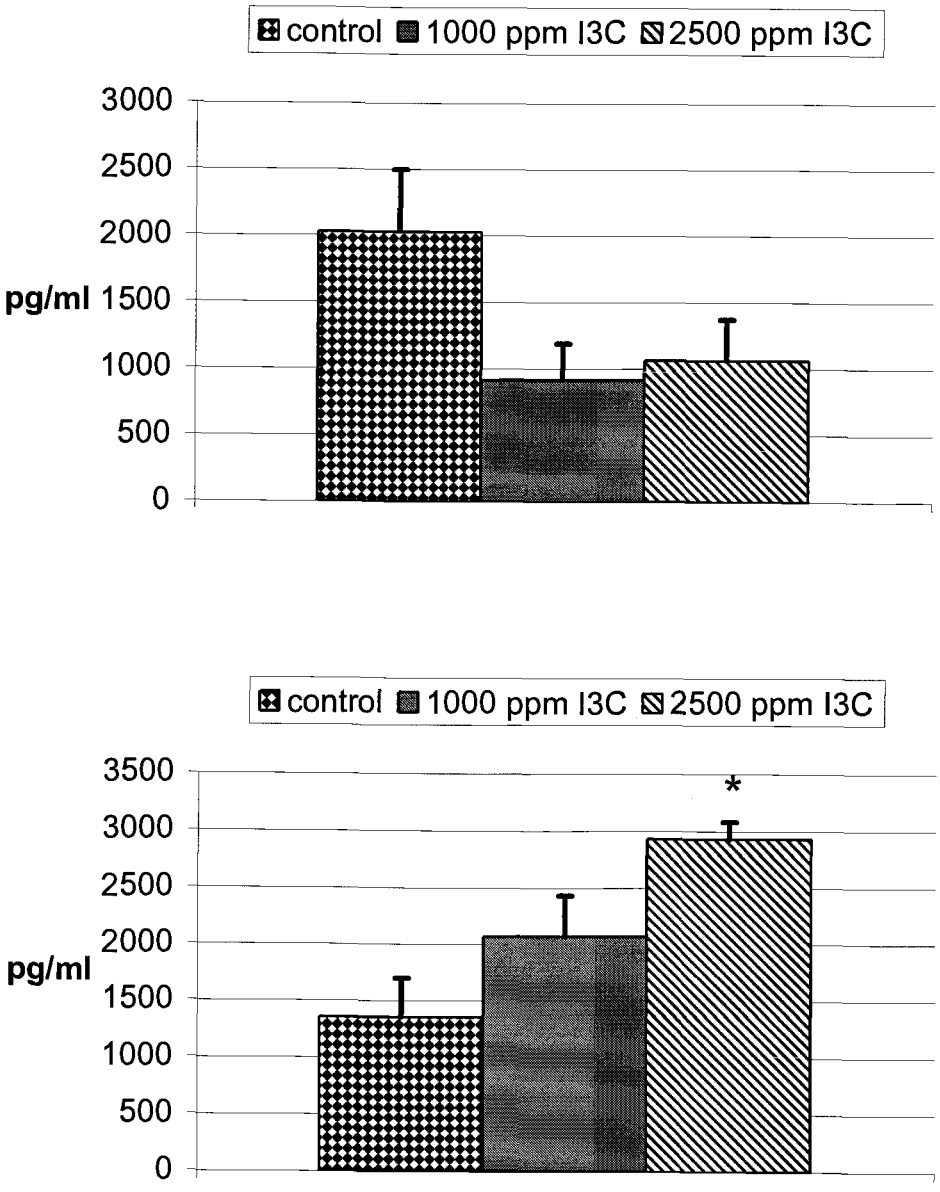

Figure 2.6: Serum testosterone levels in male rats after 6 (top) or 12 (bottom) months exposure to I3C. Values represent group averages ± SE. *$p < 0.05$ compared to corresponding control group.

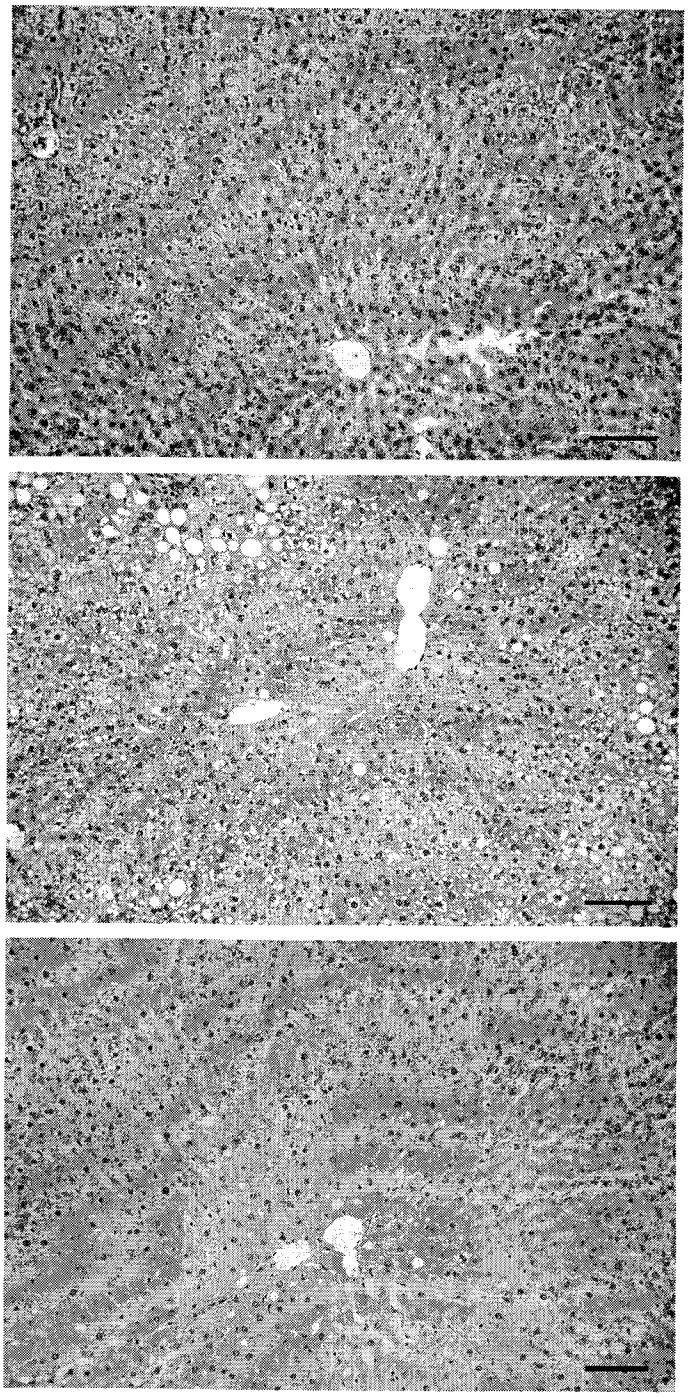

Figure 2.7: Photomicrographs of haematoxylin stained liver from rats fed control diet (top), diet containing 1000 ppm I3C (middle), and diet containing 2500 ppm I3C (bottom) for 12 months. The preparation of tissues was as described in the Materials and Methods. Bar= 50 μm.

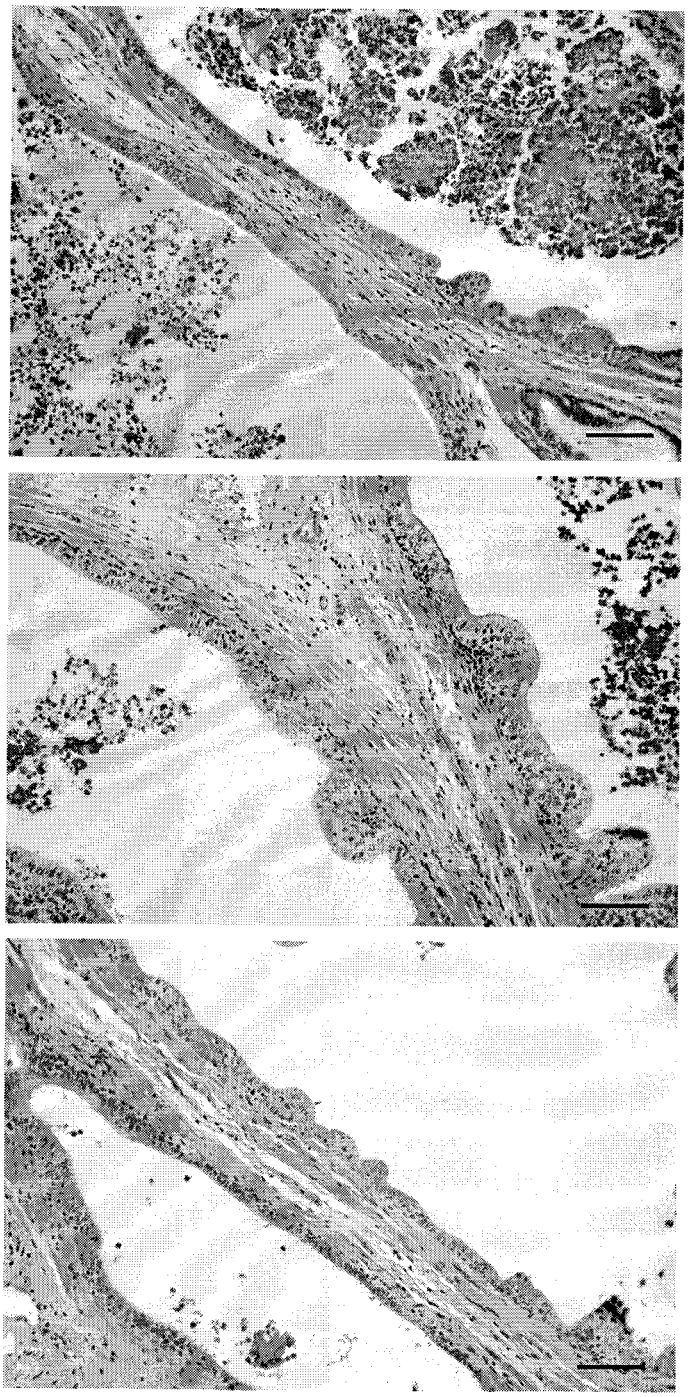

Figure 2.8: Photomicrographs of haematoxylin stained prostate from rats fed control diet (top), diet containing 1000 ppm I3C (middle), and diet containing 2500 ppm I3C (bottom) for 12 months. The preparation of tissues was as described in the Materials and Methods. Bar= 50 μm.

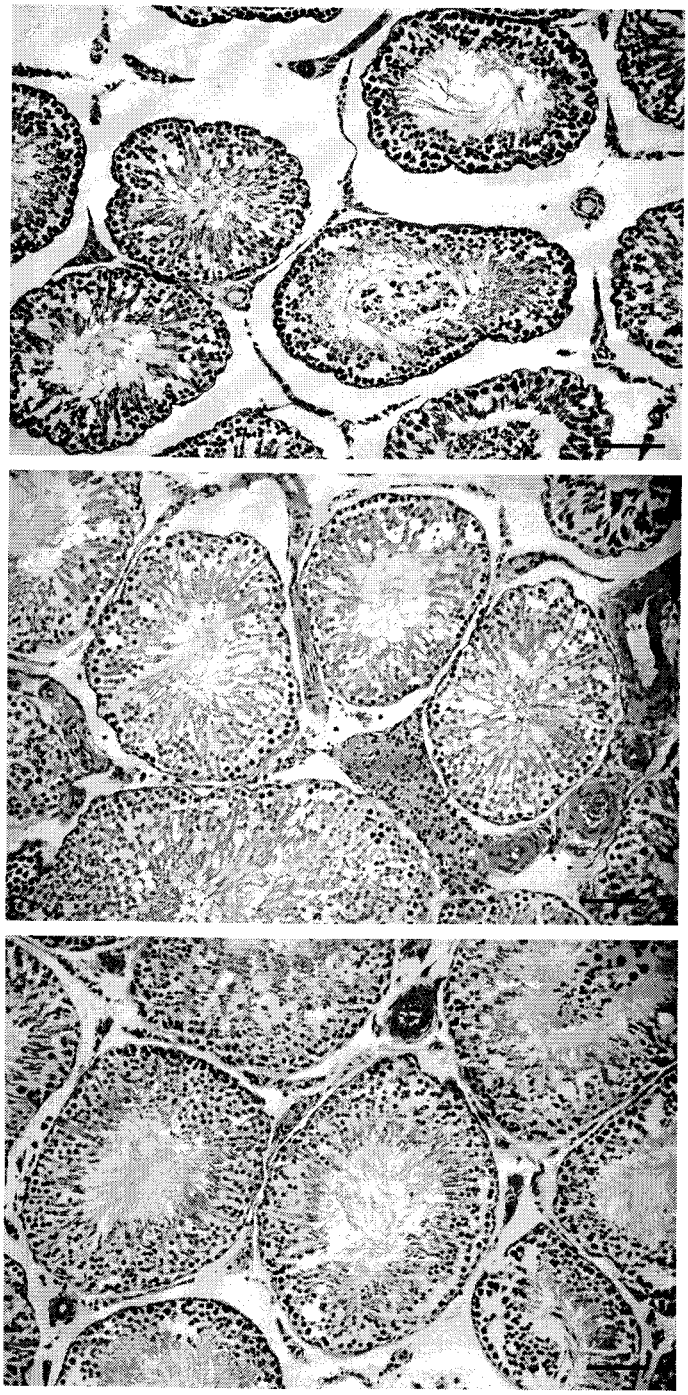

Figure 2.9: Photomicrographs of haematoxylin stained testes from rats fed control diet (top), diet containing 1000 ppm I3C (middle), and diet containing 2500 ppm I3C (bottom) for 12 months. The preparation of tissues was as described in the Materials and Methods. Bar= 50 μm.

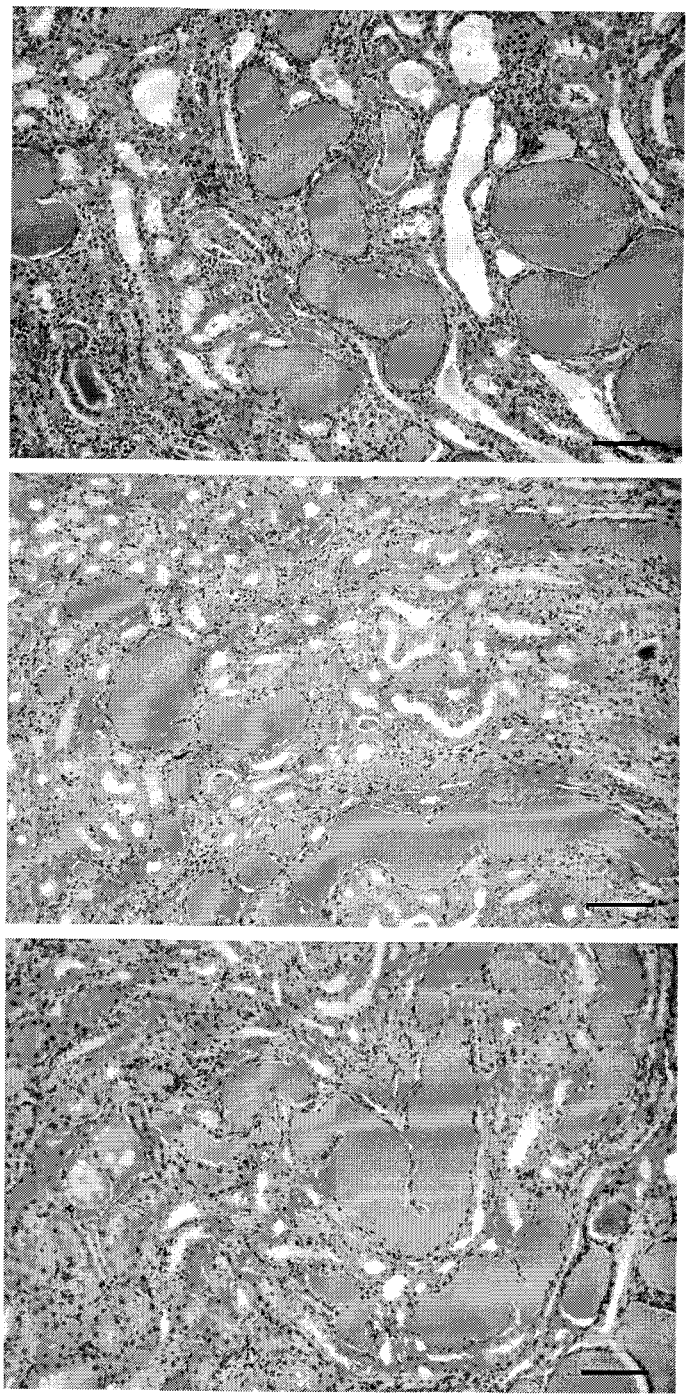

Figure 2.10: Photomicrographs of haematoxylin stained kidney from rats fed control diet (top), diet containing 1000 ppm I3C (middle), and diet containing 2500 ppm I3C (bottom) for 12 months. The preparation of tissues was as described in the Materials and Methods. Bar= 50 μm.

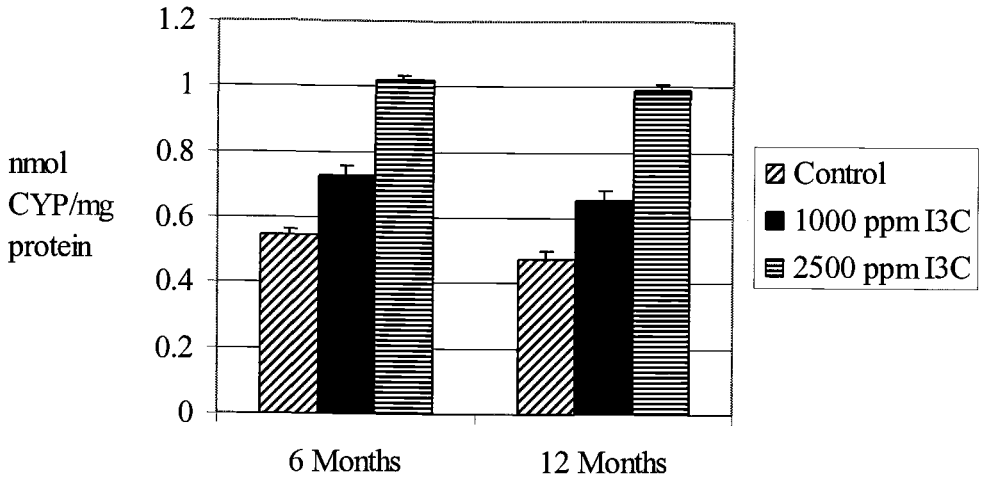

Figure 2.11: Total hepatic microsomal CYP levels in rats after 6 or 12 months of dietary I3C exposure. Values represent group averages ± SE.

CYP levels

The total hepatic CYP content of rats after 6 and 12 months exposure to I3C is shown in Figure 2.11. There was a strong increasing linear trend in total CYP with increasing I3C exposure at both time points ($p<0.0001$, Figure 2.11). I3C was also an effective inducer of CYP1A1/2 and CYP3A2 after both 6 and 12 months of exposure. Western blots and immunoquantitation of CYP1A1/2 and CYP3A2 are shown in figures 2.12 and 2.13, respectively. Relative band densities of hepatic CYP1A1/2 were increased 10- and 31- fold in rats after 6 months of I3C exposure. After 12 months CYP1A1/2 was not detected in control rats, but induction similar to the 6 month data was observed in the I3C treated groups. The band densities of hepatic CYP3A2 were elevated approximately 3- and 6- fold in rats after 6 months of exposure to 1000 and 2500 ppm I3C, respectively. After 12 months the

induction of CYP 3A2 was elevated to 5 (1000 ppm)- and 30 (2500 ppm)- fold the level found in the control group.

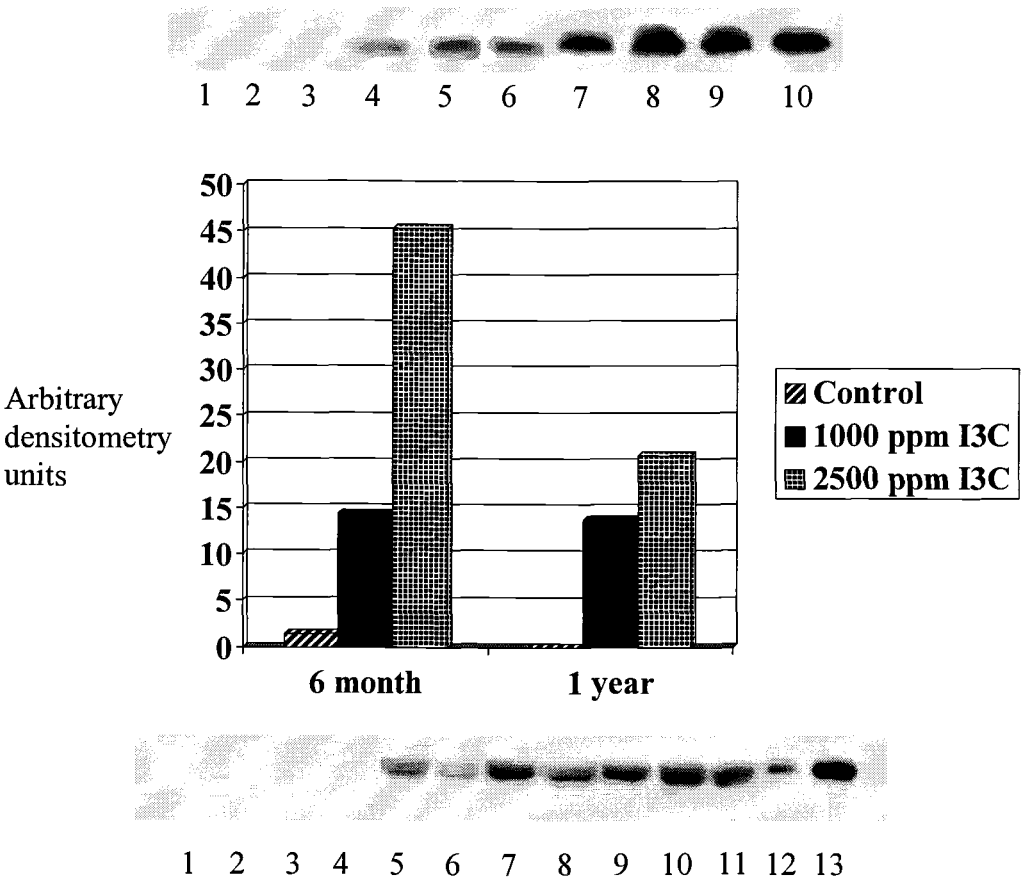

Figure 2.12: Western blots (6 month I3C exposure-top inset, 1 year I3C exposure-bottom inset) and densitometry of male rat liver microsomes for CYP1A1/1A2. Microsomal protein (22.5 μg) from rat livers was resolved by SDS-PAGE, probed with antibody to CYP1A1/1A2, and densitometry performed as described in Materials and Methods section. Lanes 1-3 (6 month) and 1-4 (1 year) represent microsomes from rats fed control diet. Lanes 4-6 (6 month) and 5-8 (1 year) represent microsomes from rats exposed to 1000 ppm I3C. Lanes 7-9 (6 month) and 9-11 (1 year) represent microsomes from rats exposed to 2500 ppm I3C. Lane 10 (4 pmol) (6 month) and lanes 12 (1.3 pmol) and 13 (3 pmol) (1 year) represent purified CYP1A1 standards.

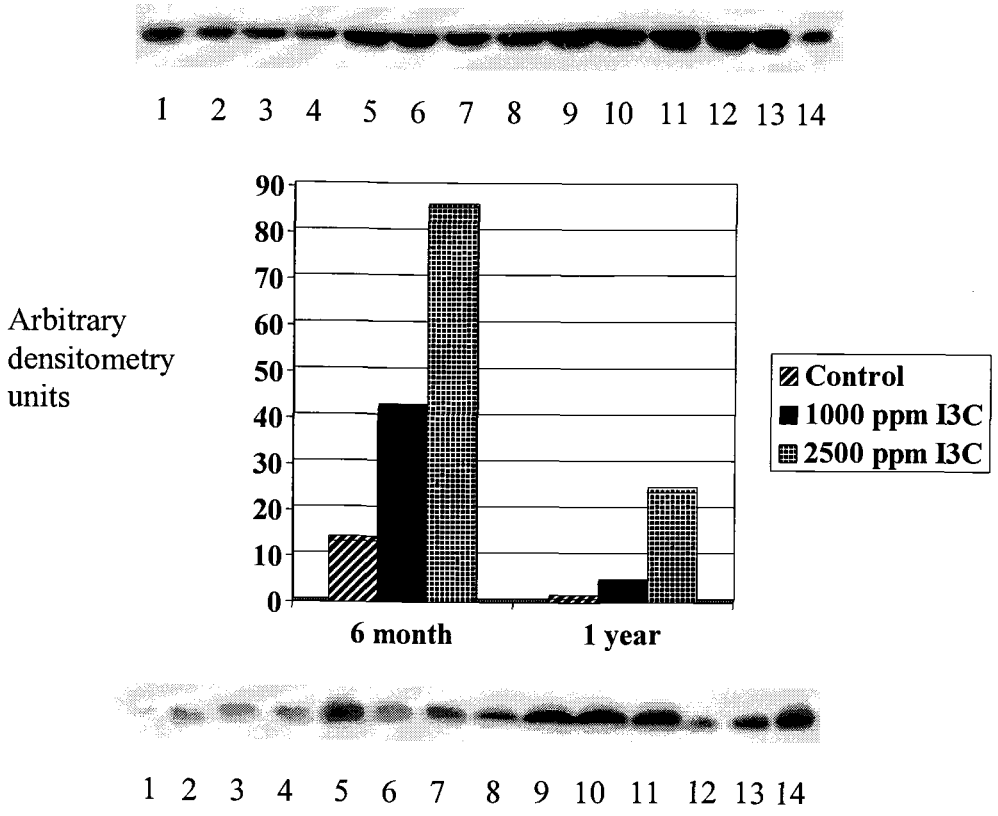

Figure 2.13: Western blots (6 month I3C exposure-top inset, 1 year I3C exposure-bottom inset) and densitometry of male rat liver microsomes for CYP3A2. Microsomal protein (21 μg) from rat livers was resolved by SDS-PAGE, probed with antibody to CYP3A2, and densitometry performed as described in Materials and Methods section. Lanes 1-4 (both blots) represent microsomes from rats fed control diet. Lanes 5-8 (both blots) represent microsomes from rats exposed to 1000 ppm I3C. Lanes 9-12 (6 month) and 9-11 (1 year) represent microsomes from rats exposed to 2500 ppm I3C. Lanes 13(.5 pmole) and 14 (0.1 pmole) (6 month blot) and 12 (.1 pmole), 13 (.25 pmole) and 14 (.5 pmole) (1 year blot) represent purified CYP3A2 standards.

Discussion

The significant evidence of a treatment related reduction in body weight for rats receiving I3C in their diet for 6 and 12 months may suggest some toxicity, especially at the 2500 ppm dose. When body weights and food consumption are considered, 2500 ppm is equivalent to approximately 60 mg/kg. These findings are consistent with data from sub-chronic studies showing that weight reduction in dogs occurred at less than 150 mg/kg (Kelloff *et al.*, 1996) and toxicities in rat appeared at doses between 20 and 2000 mg/kg. For a comparison, the daily recommended human dose for supplementation is about 10 mg/kg (Life Extensions™).

The indication of possible I3C toxicity inferred from the body weight data was not confirmed by chemistry panel analysis. Conversely, the significant reduction in alkaline phosphatase (ALP), alanine aminotrasferase (ALT), aspartate aminotransferase (AST), and creatine kinase (CK) levels after 6 months, and the significant reduction in alkaline phosphatase (ALP) levels after 12 months could indicate possible protective effects. One hypothesized potential mechanism of action of I3C as a chemopreventive agent relates to the ability of I3C to function *in vivo* as an antioxidant and as an electrophile scavenger (Shertzer *et al.*, 1988; Fong *et al.*, 1990; Arnao *et al.*, 1996; Shertzer and Senft, 2000). Therefore, in this study

continuous dietary exposure to high levels of I3C may have been inhibiting age related oxidative damage to tissues. Previous work (Dunn and LeBlanc, 1994) demonstrated that some acid condensation products of I3C lower serum LDL/VLDL cholesterol levels in mice by inhibiting acyl-CoA:cholesterol acyltransferase (ACAT). Treatment with I3C failed to provide a significant reduction of cholesterol levels in this study. With the exception of unexplained effects on globulin levels at 6 months, and calcium and amylase levels at 12 months, no other significant alterations in serum chemistry were evident.

Unpublished data from sub-chronic studies in our lab have shown decreasing levels of testosterone with increasing I3C exposure. In the current study testosterone levels appeared to follow this trend after six months but the results were not significant. This trend was reversed after 12 months of exposure when a significant increase in testosterone was observed with increasing I3C dose. Reductions in serum testosterone levels would be consistent with I3C induction of CYPs active in testosterone catabolism. The increase in testosterone levels at 12 months may be associated with the induction of CYP19 (aromatase) observed in cell cultures treated with DIM (Sanderson *et al.*, 2001). A similar induction of aromatase in the rat would lead to higher testosterone levels. We have no explanation for the impact that age appeared to have on I3C mediated effects on testosterone levels.

The absence of data indicating toxicity in the chemistry panel was confirmed by the histopathological examination. Other than the increase in hyaline casts in the kidney, no lesions were apparent in any of the tissues examined. The appearance and severity of this kidney pathology increased with age but was not treatment related.

The induction of total hepatic CYP, and the specific isoforms CYP1A1/2 and CYP3A2, observed in the current study after chronic oral adminstration of I3C, is mostly consistent with the induction seen in previous acute or sub-chronic studies (Bradfield and Bjeldanes, 1987; Bjeldanes *et al.*, 1991; Wortelboer *et al.*, 1992; Jellinck *et al.*, 1993; Stresser *et al.*, 1994; Larsen-Su and Williams, 1996; Manson *et al.*, 1997; Katchamart *et al.*, 2000). In a previous study, a seven day exposure to 0.2% dietary I3C resulted in a 24-fold induction in CYP1A1 and a 3-fold induction in CYP1A2. This is comparable to the 30-fold induction of combined CYP1A1/2 observed in this study after a chronic but similar dose of I3C. In the same seven-day study, 0.2% dietary I3C resulted in a 4-fold induction in CYP3A whereas the chronic treatment in this study resulted in a 6- and 30-fold induction after 6 and 12 months, respectively. It appears that the greater than usual induction of CYP isoforms observed after 12 months of exposure could be attributed more to an age related reduction of CYP levels in rodents (Warrington *et al.*, 2000), rather than to higher induction. The maximum induction by this dose may still be reached but the constitutive level in control rats may be less in older animals. In all cases, the

degree of induction was similar to or greater than that observed in short-term feeding studies, indicating that continuous exposure in rats does not result in any form of "tolerance" as previously demonstrated to occur in trout (Takahashi *et al.*, 1995).

Due to the fact that enzyme induction occurs continuously with exposure there is concern about the long-term effects of I3C supplementation in humans. The induction of the specific CYP isoforms by I3C may play a role in the toxicity of other compounds. The induction of CYP 1A1 and 1A2 raises concerns regarding the increased activation of polycyclic aromatic hydrocarbons such as benzo[a]pyrene and aromatic amines such as 4-aminobiphenyl. The induction of CYP 3A may be even more significant as this subfamily contributes to the metabolism of many clinically relevant drugs (Guengrich, 1999).

In this study direct toxicity of I3C was indicated only by a reduction in body weight and was not confirmed by chemistry panels or histopathology. Despite a possible protective effect against age related tissue damage, the results from this study confirm our concerns about the potential impact of dietary supplementation with I3C, with respect to alterations in drug and carcinogen metabolizing enzymes.

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CHAPTER 3

Evaluation of Chronic Dietary Exposure to Indole-3-Carbinol and Absorption-Enhanced 3,3'-Diindolylmethane in Sprague-Dawley Rats

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Abstract

Indole-3-carbinol (I3C) and 3,3'-diindolylmethane (DIM) are naturally occurring dietary components found in cruciferous vegetables. In the stomach, I3C forms condensation products including DIM. I3C and DIM are marketed as dietary supplements, but little is known about the safety of long-term exposure. Rats were fed either control diet, 1- or 10-X the current human dose of absorption-enhanced DIM or 5-7-X the maximal recommended dose of I3C. Experimental diets were fed continuously for 3 or 12 months or 2 months followed by control diet for 1 month. Results at 3 or 12 months were similar in most respects. No significant differences between groups were found in blood chemistry. A general decrease in serum enzyme levels in male rats was observed, perhaps indicative of a protective effect. Males fed I3C exhibited higher serum levels of 25-hydroxy-vitamin D_3 ($25OH\text{-}D_3$). There were no observable differences grossly or histologically between groups, although a high number of hyaline casts were found throughout the kidneys of all animals. In both sexes total hepatic CYP levels were significantly induced by I3C, but not by either dose of DIM. Induction of CYP1A1 and CYP1A2 in liver and CYP1A1 in colon was detected for both sexes fed I3C and the high dose of DIM. CYP3A2 was induced in females fed I3C or the high dose of DIM; males were induced with I3C, but not DIM. No induction of CYP1B1 in the colon was observed in either sex. Long-term exposure to DIM produced no observable toxicity and comparison to I3C indicates that DIM is a markedly less efficacious inducer of CYP in the rat at doses relevant to human supplementation.

Introduction

Indole-3-carbinol (I3C) and 3,3'-diindolylmethane (DIM) are naturally occurring plant alkaloids formed by the hydrolysis of indole glucosinolate (glucobrassicin), found in significant concentrations in cruciferous vegetables such as broccoli and Brussels sprouts (Bradfield and Bjeldanes, 1987; Slominski and Campbell, 1987; McDanell *et al.,* 1988; Preobrazhenskaya *et al.*, 1993). Furthermore, I3C is an unstable compound that undergoes rapid oligomerization in the acid environment of the stomach to form dimers, trimers, tetramers, and several other higher order condensation products (Leete and Marion, 1953; Bradfield and Bjeldanes, 1987; Bjeldanes *et al.*, 1991; Wortelboer *et al.,* 1992). The major product formed *in vitro* (Spande, 1979) and *in vivo* after oral administration (Dashwood *et al.,* 1989; Stresser *et al.*, 1995) is DIM.

Both I3C and DIM are marketed as dietary supplements and are under investigation as potential chemopreventive agents. I3C requires conversion to these acid condensation products in order to be chemopreventive against a wide variety of carcinogens and is especially effective when administered before or during the initiation phase of carcinogenesis (Bradlow *et al.*, 1999; Shertzer and Senft, 2000; Murillo and Mehta, 2001). DIM inhibits aflatoxin B_1-DNA binding in trout (Dashwood *et al.*, 1994) and mammary tumor growth in the rat (Wattenberg and Loub, 1978; Chen *et al.*, 1998).

Among the hypothesized mechanisms of chemoprevention of I3C and DIM is their ability to modulate xenobiotic metabolizing enzymes and induce estrogen

metabolism. When administered through the diet in short-term studies, I3C and DIM induce a number of phase I enzymes in liver and colon, including cytochrome P450 (CYP) 1A1, CYP1A2, and CYP 3A (Vang *et al.*, 1990; Jellinck *et al.*, 1993; Stresser *et al.*, 1994; Bonnesen *et al.*, 2001; Horn *et al.*, 2002;). Increased activity of phase I drug-metabolizing enzymes can protect against some carcinogens by increasing their rate of oxidative metabolism to less toxic metabolites (Park and Bjeldanes, 1992; Stresser *et al.*, 1994; Xu *et al.,* 1997; He *et al.*, 2000) however, an increase in activity of certain CYP isozymes could enhance carcinogenicity of some chemicals by increasing their rate of bioactivation (Ioannides and Parke, 1993).

I3C- and DIM- dependent alterations of the monooxygenase systems also raise concerns relative to their potential adverse effects on drug/xenobiotic metabolism. Studies have shown differential metabolism of tamoxifen and nicotine by liver microsomes from rats fed I3C (Katchamart *et al.*, 2000). I3C induction of CYPs in the 1 family markedly enhances estradiol metabolism. CYP1A1 and 1A2 catalyze the 2-hydroxylation of β-estradiol (E_2), whereas CYP1B1 is an effective E_2-4-hydroxylase (Hayes *et al.*, 1996). Both of these metabolites are catechol estrogens and represent potential toxic metabolites. Evidence suggests that 4-OH-E_2 is the more reactive and toxic metabolite (Newbold and Liehr, 2000).

Preliminary evidence to date indicates that DIM maybe a safer alternative to I3C as it is relatively stable in acid and does not undergo further condensation reactions, preventing the formations of toxic metabolites such as indolo[3,2-*b*]carbazole (ICZ), a potent aryl hydrocarbon receptor agonist (Bjeldanes *et al.*,

1991) and potential promoter of hepatocarcinogenesis in the rat (Herrmann *et al.*, 2002). DIM induces apoptosis and inhibits growth of human cancer cells (Ge *et al.*, 1996; Chen *et al.*, 2001; Leong *et al.*, 2001; Hong *et al.*, 2002) and inhibits estrogen-dependent mammary tumorigenesis in rats (McDougal *et al.*, 2001). However, due to poor bioavailability of DIM, its use as a supplement has been limited. The objective of this study was to evaluate the biological effects of long-term exposure to rats of an absorbable formulation of pure DIM, (BioResponse DIM® (Indolplex®)) at the current maximal human dose (US Patent #6,086,915; Zeligs *et al.*, 2002; Anderton *et al.*, 2003). In addition, a pharmacological dose 10 times the current human dose was tested for toxicity and, as a comparison, to a similar and previously studied dose of I3C.

Materials and Methods

Chemicals

Indole-3-carbinol was obtained from Sigma-Aldrich Co. (Milwaukee, WI). 3,3'-Diindolylmethane was provided in a bioavailable formulation (BioResponse-DIM®) by BioResponse, LLC (Boulder, CO). The DIM content was independently verified by HPLC (Eurofins-Alpha Laboratories, Petaluma, CA).

Animals

One hundred and forty Sprague-Dawley rats (70 each sex) were purchased from Simonsens (Gilroy, CA) at four weeks of age. After a one-week acclimation period, animals were randomly divided into 10 different treatment groups, each containing 7 rats of each sex (Table 3.1). Rats were housed individually in hanging metal wire cages at the Laboratory Animal Resource Center, Oregon State University, and maintained at 22°C and 40 to 60 % humidity on a 12 hour light/dark cycle. Both tap water and powdered semisynthetic diet were available *ad libitum* throughout the study. Groups 1 and 7 received only control (AIN-76A) diet. The diet for groups 2, 5 and 8 was supplemented with I3C to levels providing a dose of 50 mg/Kg/day. The diets are prepared by thoroughly mixing the indoles as a powder into the powdered AIN76A diet. The diets were made weekly and stored at 4°C in sealed containers protected from light. I3C is known to be unstable, especially under acidic conditions. Although we did not assay I3C and DIM in the diets used in this study, a previous study involving long-term feeding of mice with AIN76A diet demonstrated that under these same conditions at least 80%

TABLE 3-1. STUDY DESIGN

Group	Duration	Supplement	Daily Dose	Males	Females
1	3 months	-	None	7	7
2	3 months	I3C	50 mg/Kg	7	7
3	3 months	DIM	2 mg/Kg	7	7
4	3 months	DIM	20 mg/Kg	7	7
5	2 months 1 month	I3C -	50 mg/Kg None	7	7
6	2 months 1 month	DIM -	20 mg/Kg None	7	7
7	12 months	-	None	7	7
8	12 months	I3C	50 mg/Kg	7	7
9	12 months	DIM	2 mg/Kg	7	7
10	12 months	DIM	20 mg/Kg	7	7

Four week old Sprague-Dawley rats were obtained commercially, acclimated for 1 week and then randomly assigned to 1 of the 10 treatment groups. The housing, preparation of diet and experimental details are described in Materials and Methods.

of the I3C was present after one week (Oganesian et al., 1997). The stability of DIM is expected to be much greater as we have found it to be relatively stable under mild acid conditions in air at room temperature (unpublished observations). The diets for groups 3, 4, 6, 9 and 10 provided 6.6 mg/Kg/day or 66 mg/Kg/day BioResponse DIM™. On a molar basis, the daily doses of I3C and low and high DIM were 0.34, 0.008 and 0.08 mmol/Kg, respectively. The approximately 2.5 and 25% molar levels of DIM (relative to I3C) span the range for the percentage of DIM found after acid condensation reaction of I3C *in vitro* or *in vivo*.

Rats were weighed and food consumption estimated weekly for the first three months and monthly thereafter. The groups were euthanized after either 3 (groups 1-6) or 12 months (groups 7-10) of treatment. In order to determine if the effects of dietary I3C or DIM were reversible, rats in groups 5 and 6 were fed I3C or high DIM for 2 months and then switched to control AIN76A diet for 1 month prior to euthanasia. At the end of the exposure period rats were weighed and all diets were removed overnight before sampling. Rats were anesthetized and blood samples were collected as described in the following section. While remaining under anesthesia, rats were euthanized by exsanguination. Gross pathology was performed and relevant tissue (see below) weights were recorded. A portion of the liver, colon, kidneys, adrenal, uterus and ovaries for females and liver, colon, kidneys, adrenal, prostate, testicles, and epididymus for males was preserved in neutral buffered formalin for subsequent sectioning and histopathology. A second portion of the liver and colon were removed prior to fixation and immediately

frozen in liquid nitrogen and then stored at -80° C until analysis. The carcasses of rats were stored at 1.5°C for bone density analysis. The above protocols were approved by the Oregon State University Institutional Animal Care and Use Committee.

Blood collection and analysis

Blood was collected from the abdominal aorta while rats were anesthetized under 4% isoflurane with 0_2 at a flow of 2 L/min. Blood was stored at 4°C for 2 hours and then spun for 20 minutes at 11,000 rpm to isolate serum. One aliquot of serum was sent to the Texas Veterinary Medical Diagnostic Laboratory (College Station, TX) where a small animal clinical chemistry analysis was performed utilizing a Hitachi 911 Chemical Analyzer (Roche, Indianapolis, IN). A second aliquot was sent to the Animal Health Diagnostic Laboratory (Lansing, MI) where circulating 25-hydroxyvitamin D_3 (25-OH-D_3) levels were determined using a commercial radioimmunoassay from DiaSorin (Stillwater, MN) and testosterone levels were determined (males only) using a commercial radioimmunoassay from Diagnostic Products Corporation (Los Angeles, CA).

Bone density analysis

Three carcasses of each sex, from groups 1, 2, and 4 were sent on ice to University Colorado Health Science Center (William. E Huffer, M.D., Denver, CO). The animals were equilibrated to 4° C and the proximal knee joint with approximately one half of the distal femur and proximal tibia were removed and fixed for 24 hours in absolute methanol also at 4°C. The specimens were then

embedded in glycol methacrylate, and sectioned on a rotary microtome with a D-profile carbon-tungsten steel knife at 5 μm. The sections were stained by the von Kossa technique (Bills *et al.,* 1971) with a hematoxylin and eosin counter-stain to demonstrate general histology and calcified bone and osteoid, and for tartrate resistant acid phosphatase to demonstrate osteoclasts on bone surfaces. Measurements of the total and cancellous bone areas, cortical bone thickness, and the number of osteoclasts in a single section of the tibial metaphysis were made by semi-automated histomorphometric methods (Huffer and Lepoff, 1992; Huffer *et al.,* 1994).

Histopathology

Tissues were fixed in 10% neutral buffered formalin and processed on a LX300 Tissue Processor (Fisher Scientific). Sections were cut at 4-5 microns and stained on the S/P Automatic Slide Stainer GLX with Haematoxylin (Gill-3, Shandon Inc.-Pittsburgh, PA) and Eosin Y (alcoholic, Shandon Inc.-Pittsburgh, PA). Slides were coverslipped with Shur/Mount (Triangle Biomedical Sciences-Durham, NC).

Microsome preparation and total CYP

Liver samples were homogenized with a Brinkmann Polytron (Westbury, NY) into 3 volumes of cold homogenization buffer (10 mM potassium phosphate [pH 7.5] containing 0.15 M potassium chloride, 1 mM EDTA, and 0.1 mM phenylmethylsulfonylfluoride (PMSF)). Microsomes were prepared by ultracentrifugation according to Guengerich (1989) and protein levels were

determined by the method of Lowry *et al.* (1951). The total liver microsomal CYP content was quantified by the CO versus CO-reduced difference spectra (Omura and Sato, 1964) on a Cary 300 UV-Vis spectrophotometer (Varian, Walnut Creek, CA).

Colon lysate preparation

Colons were rinsed with phosphate buffered saline (PBS) to remove mucus and then scraped with the back of a scalpel blade to remove cells. Cells were lysed in a modified RIPA buffer (1x PBS, 1.0% Igepal CA-630, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) with 1 tablet of Complete-Mini protease inhibitor cocktail (Roche, Indianapolis, IN) added for every 10 ml of buffer) and then sheared by passing through a 23 gauge needle. Sheared cells were incubated 45 min on ice after the addition of 0.574 mM PMSF (1:100 from an isopropanol stock) and then spun at 10,000 rpm for 10 min at 4°C. The supernatant was removed, protein concentration determined by the method of Lowry *et al.* (1951) and stored at -80°C until analysis.

Electrophoresis and immunoblotting

Microsomal and lysate proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and electrophoretically transferred to nitrocellulose membrane (Towbin *et al.*, 1979). The membranes were incubated with goat antibodies recognizing both rat CYP1A1 and CYP1A2 or CYP3A2 (Gentest, Woburn, MA) and probed with rabbit anti-goat secondary antibody conjugated with horseradish peroxidase (Kirkegaard & Perry Laboratories,

Gaithersburg, MD) or rabbit antibody raised against rat CYP1B1 (Gentest, Woburn, MA) and probed with goat anti-rabbit secondary antibody also conjugated to horseradish peroxidase (Gentest, Woburn, MA). The blots were visualized by a chemiluminescence detection kit (New England Nuclear, Boston, MA) and densitometry was performed using an HP Scanjet IIcx flatbed scanner and NIH Image software version 1.61/ppc (public domain, National Institutes of Health).

Statistical analysis

Males and females were analyzed separately. When assumptions were reasonably satisfied, treatments were compared using one-way ANOVA and all pairwise comparisons with Tukey's multiple comparison adjustment. When outliers or other non-normality were indicated, simple transformations, such as logarithmic, were examined. When the problem was non-transformable, non-parametric rank tests (Kruskal-Wallis) were used. In that case pairwise comparisons were adjusted for multiple comparisons using the global permutation distribution, after first determining that there were no problems with extreme heteroscedasticity or badly unbalanced sample sizes (Westfall *et al.,* 1999). When preliminary analysis indicated normality but heterogeneity of variance, a general mixed model allowing heterogeneity of variance was fit by residual maximum likelihood (REML) with Tukey adjusted pairwise comparisons. The denominator degrees of freedom for testing were adjusted by the method of Kenward and Rogers (SAS, 1999). Statistical analyses were conducted using SAS version 8.2 (Cary,

NC). Within SAS/STAT the GLM, Mixed, Npar1way and Multtest procedures were used.

Results

Body and tissue weights

There were no significant differences in diet consumption or weight gain between groups. The body weights in both sexes tended to be lower in the I3C-fed group, but the analysis of weights at the time of sacrifice revealed no significant differences between treatments (p=0.23 and 0.44, ANOVA, for males and females, respectively, Figure 3.1). There were also no treatment related effects on raw tissue weights in either sex, although I3C, but not DIM significantly increased the liver somatic index (LSI) in males at both the 3 and 12 month time points (Table 3.2). In the group fed I3C for 2 months followed by control diet for 1 month, the LSI was lower relative to the group fed I3C for 3 months, but the difference was not statistically significant. Both sexes fed I3C had increased LSI after 12 months relative to controls, but only the increase in males was large enough to be statistically significant after multiple comparison adjustment (p=0.0023 t-test, (Table 3.2) and p=0.086 Wilcoxon for males and females, respectivly). The high dose of DIM marginally induced LSI in males after 12 months (p=0.042, Tukey-adjusted t-test), but not in females.

Clinical chemistry, 25OH-D_3 and testosterone levels

The results of the clinical chemistry panel with female rats did not demonstrate any treatment related effects after 3 or 12 months of exposure, with the exception of phosphorus in females fed the high DIM diet (3.17 mg/dl compared to 4.20 in controls, adjusted p value=0.0279, Table 3.3). In males, neither I3C nor DIM

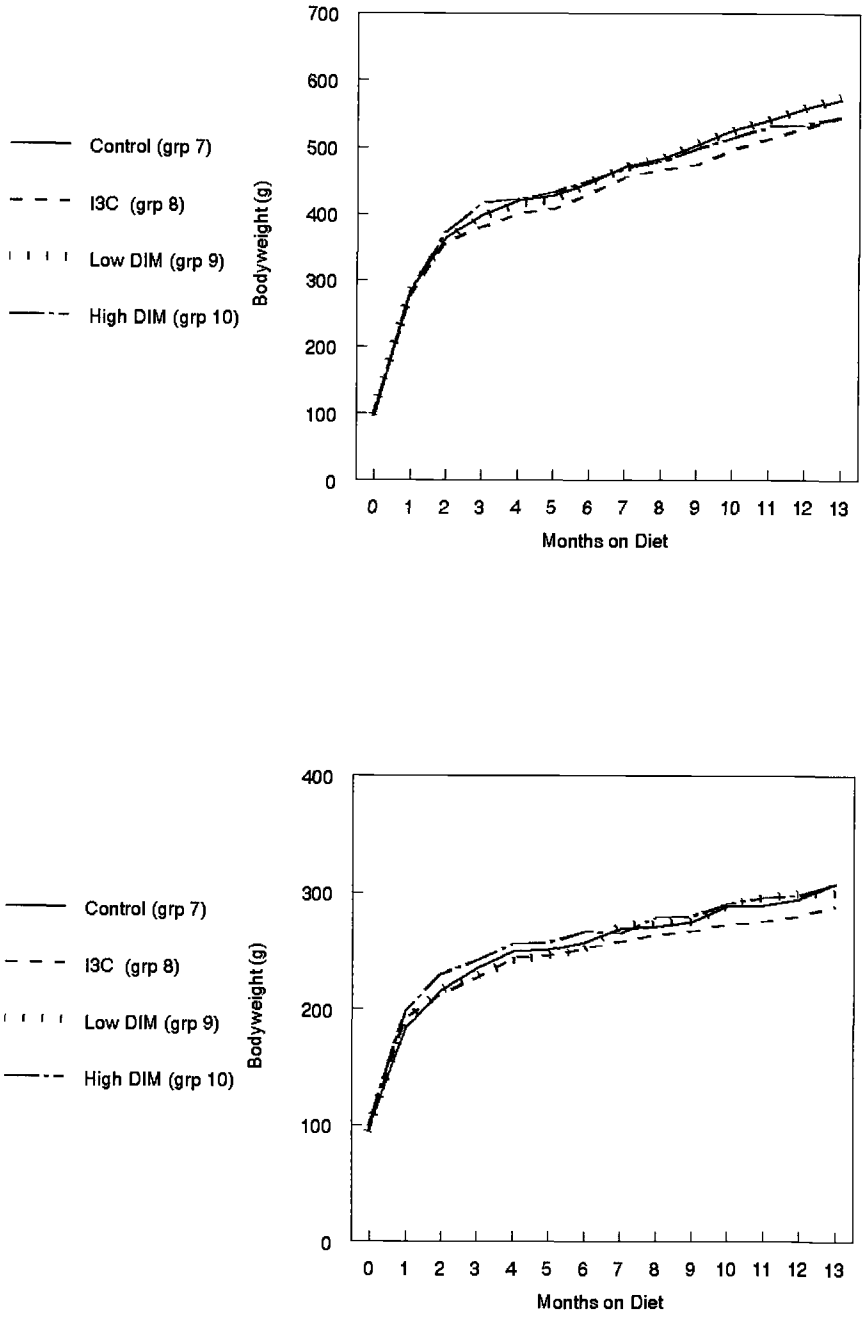


Figure 3.1: Body weights of male (top) and female (bottom) rats fed control, I3C or DIM for 12 months.

TABLE 3.2. LIVER SOMATIC INDEX AT 3 AND 12 MONTHS

Group	Treatment	LSI (± SD)
1	3 month control	3.58±0.25
2	3 month I3C	4.00±0.45*
3	3 month low DIM	3.58±0.15
4	3 month high DIM	3.66±0.25
5	2 month I3C/1 month control	3.74±0.26
6	2 month DIM/1 month control	3.65±0.26
7	12 month control	2.51±0.18
8	12 month I3C	2.93±0.08*
9	12 month low DIM	2.65±0.23
10	12 month high DIM	2.82±0.24*

LSI= Liver weight/body weight x 100. The results are the mean (± SD).
* Indicates statistical significance at $p<0.05$

elevated biomarkers of tissue damage, and in fact a general decrease in the levels of serum enzymes after 12 months of experimental diet administration was observed in male rats (Figure 3.2). Serum aspartate aminotransferase (AST) levels were significantly lower in male rats fed I3C or either dose of DIM for 12 months ($p<0.013$, for each Tukey-adjusted comparison). A heterogeneous variance mixed model was used for analysis because rats in the control group exhibited greater variation than the other groups. Although alanine aminotransferase (ALT) levels at 12 months were also lower in these same rats, the difference, relative to controls, was not found to be statistically significant due to high variability in the control group ($p>0.11$, all adjusted pairwise comparisons). Alkaline phosphatase (ALP) serum levels were reduced in male rats fed I3C ($p=0.010$) and the high dose

TABLE 3.3. CLINICAL CHEMISTRY FOR FEMALE RATS

Group	Serum Protein	Albumin	Calcium	Phosphorous	Glucose	BUN
1	5.9±.16	3.8±.21	10.2±.18	5.0±.26	202±9.1	19±.9
2	6.0±.10	4.1±.17	10.1±.25	4.8±.42	207±11.4	20±1.4
3	5.8±.10	4.1±.07	10.1±.12	5.2±.40	194±2.7	19±1.2
4	5.7±.08	4.1±.04	10.0±.06	5.1±.25	195±8.8	19±.6
5	5.8±.11	4.0±.11	10.0±.06	4.4±.23	200±6.9	20±1.0
6	5.9±.11	4.0±.13	9.9±.09	4.8±.31	193±7.3	19±.8
7	5.6±.30	3.9±.24	11.0±.23	4.2±.23	189±9.8	19±.9
8	5.3±.30	3.8±.19	10.6±.16	4.0±.18	179±3.9	20±.9
9	5.4±.27	3.5±.25	10.5±.19	3.5±.25	202±10.0	17±.7
10	6.2±.06	4.4±.07	11.3±.12	3.2±.28*	171±9.3	19±1.0

Group	Creatinine	Bilirubin	Globulin	GGT	Amylase	Cholesterol
1	0.14±.02	0.1±0	2.1±.32	<3	1450±88	101±5
2	0.17±.03	0.1±.01	1.9±.10	<3	1673±142	114±3
3	0.17±.02	0.1±0	1.8±.06	<3	1811+284	109±4
4	0.13±.02	0.1±.01	1.6±.05	<3	1334±38	102±4
5	0.17±.03	0.1±.01	1.8±.09	<3	1685±121	111±5
6	0.14±.02	0.1±0	2.0±.18	<3	1479±66	101±2
7	0.2±.03	0.2±.01	1.7±.06	<3	1212±133	152±15
8	0.17±.02	0.2±.01	1.6±.12	<3	1019±89	148±10
9	0.24±.03	0.2±0	1.9±.28	<3	1111±110	129±8
10	0.22±.02	0.2±.02	1.8±.06	<3	1216±49	147±5

* = $P < 0.05$

of DIM (p=0.004). The only significant reduction in creatinine kinase (CK) was in the male rats fed the high dose of DIM (p=0.010). At 12 months there were no significant differences between control and treatment groups in either sex for any of the remaining serum chemistry measurements (total protein, albumin, calcium, glucose, BUN, globulins, GGT, cholesterol, amylase, bilirubin, creatinine)(Table 3.3 and 3.4).

TABLE 3.4. CLINICAL CHEMISTRY FOR MALE RATS

Group	Serum Protein	Albumin	Calcium	Phosphorous	Glucose	BUN
1	5.9±.10	3.8±.09	10.0±.12	6.1±.26	194±10	21±1.2
2	5.9±.08	3.9±.05	9.8±.15	6.4±.22	194±4	18±.6
3	5.9±.04	3.8±.03	10.2±.08	6.5±.27	199±8	16±1.4
4	5.8±.06	3.9±.07	10.1±.22	6.8±.16	189±11	19±0.8
5	5.8±.10	3.7±.06	10.1±.12	6.5±.18	202±14	18±1.5
6	5.7±.11	3.8±.06	9.8±.15	6.5±.28	203±12	19±1.0
7	6.1±.11	3.4±.12	11.4±.19	4.5±.29	199±31	26±7.8
8	5.8±.29	3.5±.20	11.3±.44	4.1±.29	206±21	17±0.8
9	5.9±.09	3.4±.08	11.7±.17	4.2±.21	195±14	24±4.1
10	5.5±.30	3.2±.19	11.5±.24	4.1±.31	198±14	29±7.4

Group	Creatinine	Bilirubin	Globulin	GGT	Amylase	Cholesterol
1	0.12±.02	0.1±0	2.0±.04	<3	2565±132	96±4
2	0.12±.01	0.1±0	2.1±.04	<3	2736±121	116±5
3	0.12±.01	0.1±0	2.1±.04	<3	2641±70	103±5
4	0.10±0	0.2±.02	1.9±.05	<3	2745±126	110±6
5	0.14±.02	0.1±.01	2.1±.12	<3	2665±163	108±6
6	0.10±0	0.1±0	2.0±.05	<3	2508±89	101±4
7	0.49±.16	0.2±.03	2.7±.12	<3	2233±79	239±29
8	0.17±.03	0.1±.02	2.3±.14	<3	2392±138	274±25
9	0.34±.06	0.1±.01	2.5±.14	<3	2720±163	288±32
10	0.42±.17	0.1±.02	2.3±.14	<3	2747±234	315±24

Serum 25-OH-D_3 levels were increased ($p=0.0371$ and 0.0031 for males and females, respectively) about 50% by dietary I3C in both sexes after 12 months. At 3 months only the males exhibited elevated serum levels of 25-OH-D_3. Again, the I3C diet increased levels by about 50%. The high dose of DIM also appeared to increase 25-OH-D_3 in serum, but the change was not significant (data not shown).

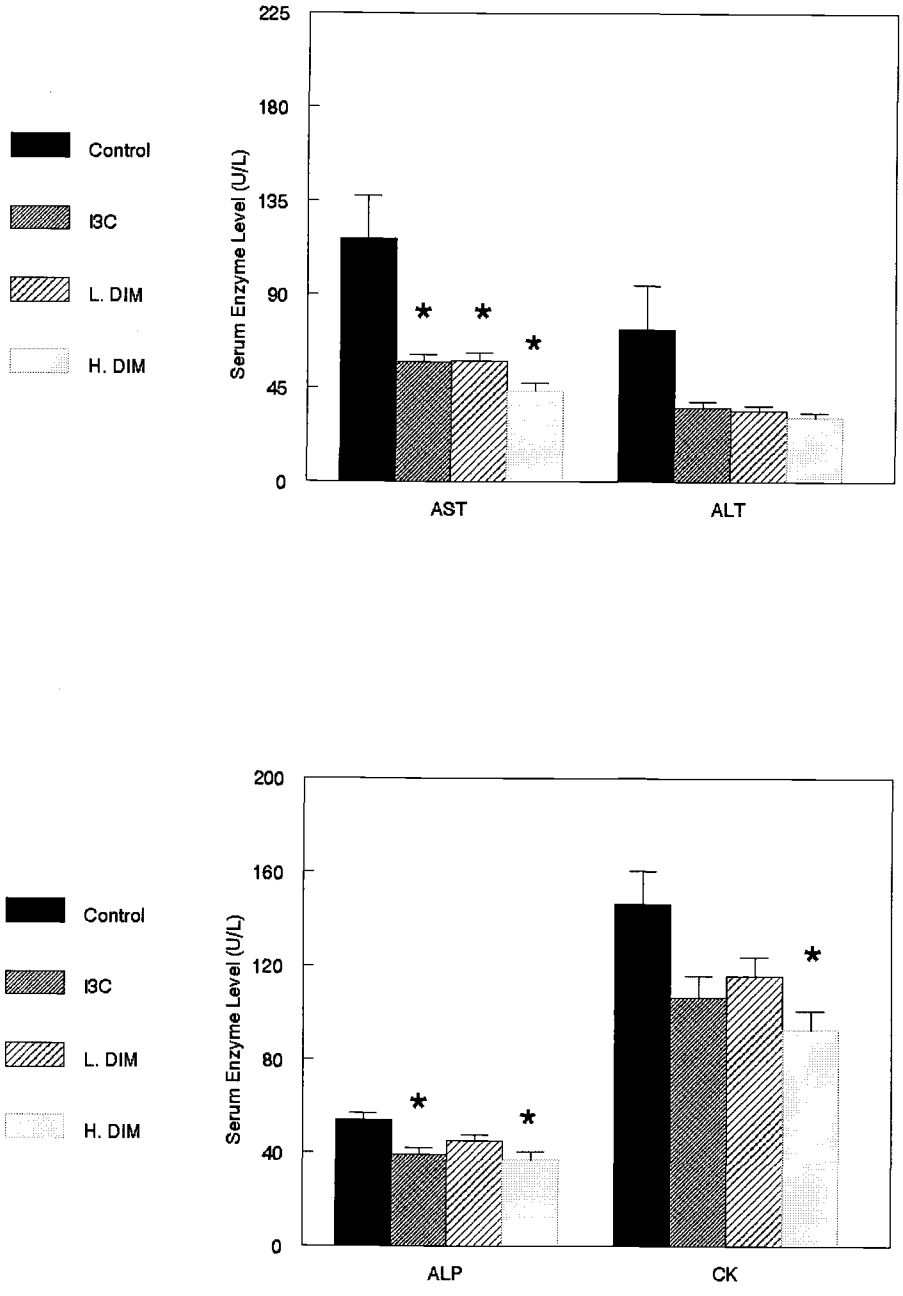


Figure 3.2: Serum enzyme levels in males after 12 months exposure to I3C or DIM. AST and ALT (Top); ALP and CK (Bottom). Values represent group averages in U/L $\pm$ SE. * = $p<0.05$.

Serum testosterone levels in males were not altered by test diets. E_2 levels were unchanged by I3C or DIM at both the 3 and 12 month time points (data not shown).

Histopathology

No significant differences between groups of either sex at 12 months were noted upon necropsy or following histopathology. Most notably, no effects were seen in hormone responsive tissues such as prostate in male rats (Figure 3.3) and ovaries in female rats (Figure 3.4), and no toxicities were indicated in treatment groups as compared to controls in the liver (Figures 3.5 and 3.6). There were numerous, large hyaline casts found in the kidney tubules. The appearance of such casts is common in rats as they age (Lord and Newberue, 1990), and no treatment related differences were evident (Figures 3.7 and 3.8).

CYP levels

The total hepatic CYP of the male and female rats at 12 months is shown in Figure 3.9. A significant increase in total CYP was observed in the I3C supplemented groups of both sexes. DIM did not significantly induce total hepatic CYP in either sex.

As previously demonstrated by our laboratory and others (Bradfield and Bjeldanes, 1987; Bjeldanes *et al.*, 1991; Wortelboer *et al.*, 1992; Stresser *et al.*, 1994; Larsen-Su and Williams, 1996; Manson *et al.*, 1997; Katchamart *et al.*, 2000) I3C effectively induces CYP1A1 and 1A2 levels in liver microsomes following oral administration. After 3 months, CYP1A1/1A2 was induced 20- and 44-fold in rat liver microsomes from males and females, respectively. DIM also significantly

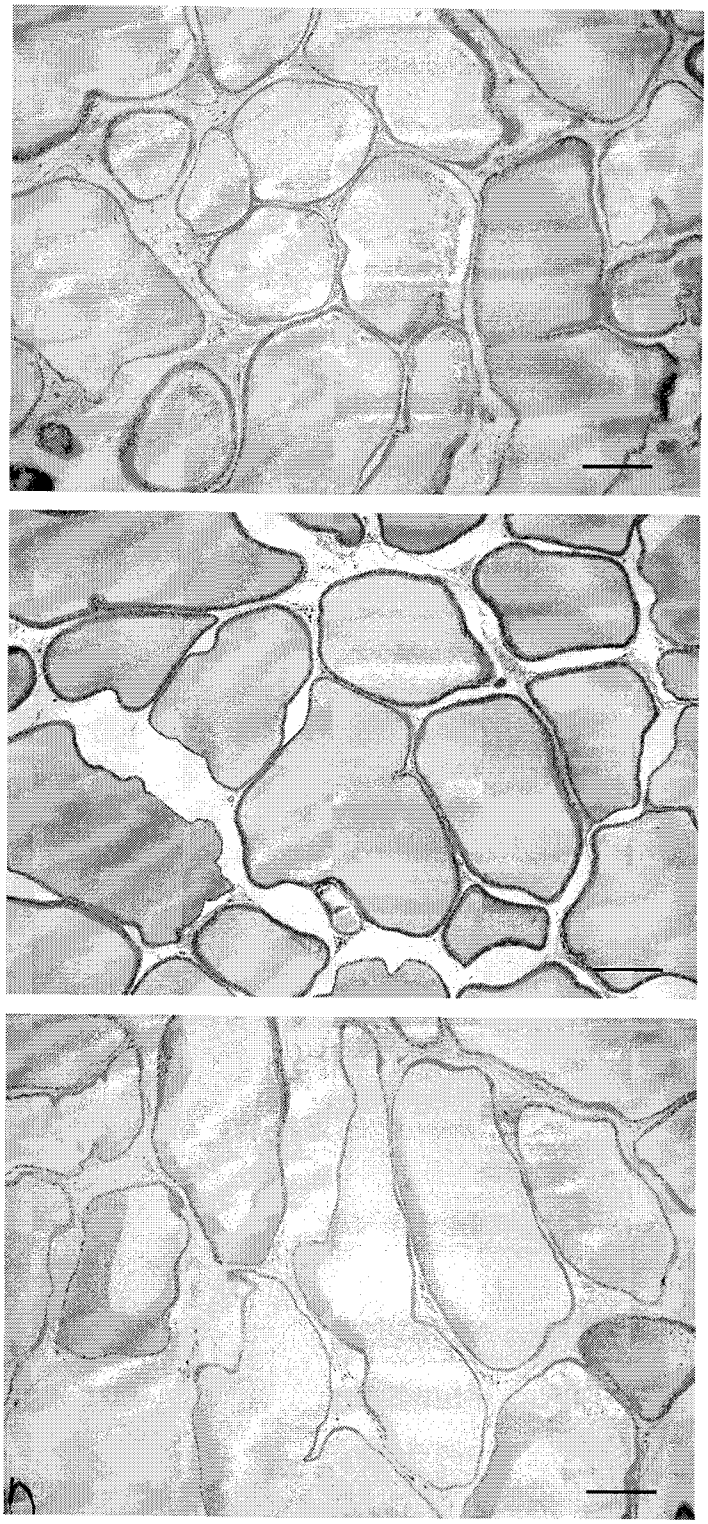


Figure 3.3: Photomicrographs of haematoxylin stained prostate from control (top), I3C (middle), and DIM (bottom) treated rats at 12 month time point. The preparation of tissues was as described in the Materials and Methods. Bar= 50 μm.

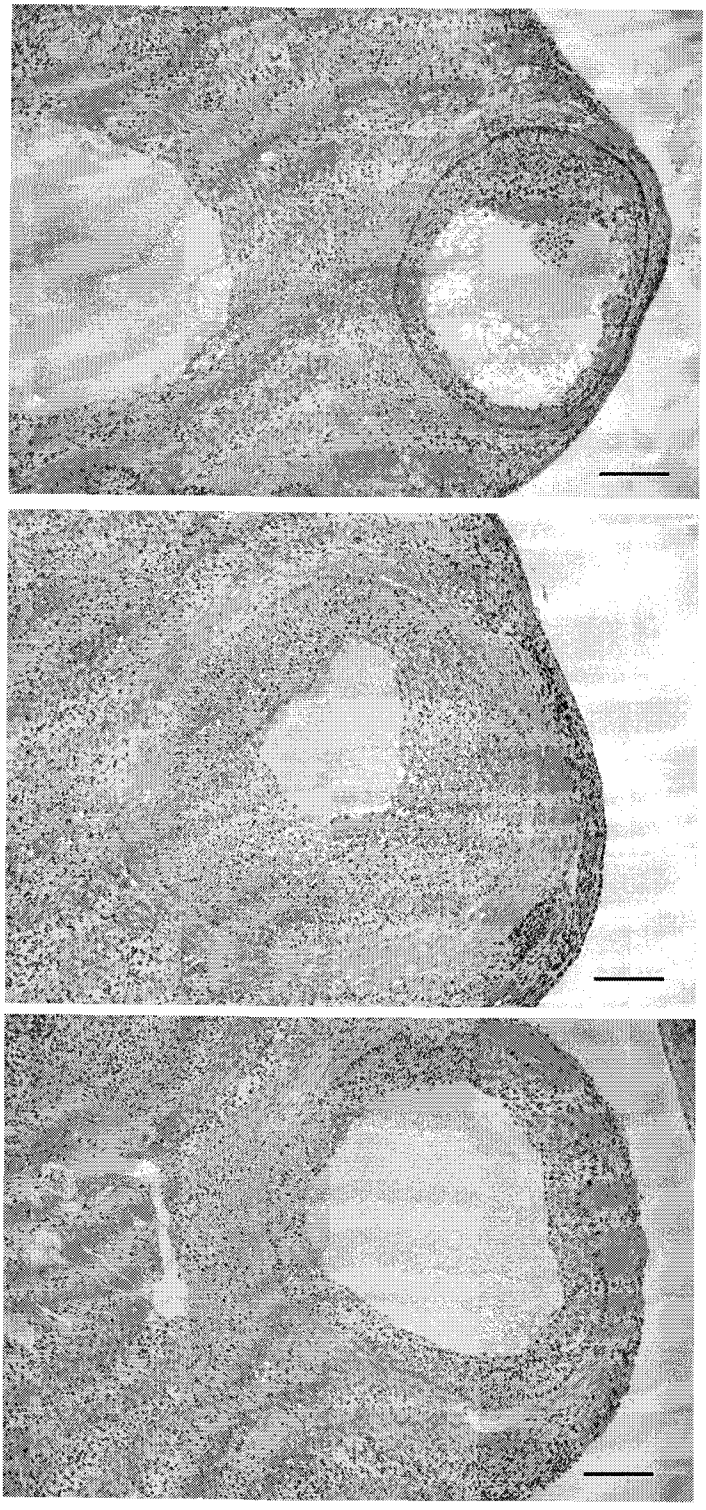


Figure 3.4: Photomicrographs of haematoxylin stained ovary from control (top), I3C (middle), and DIM (bottom) treated rats at 12 month time point. The preparation of tissues was as described in the Materials and Methods. Bar= 50 μm.

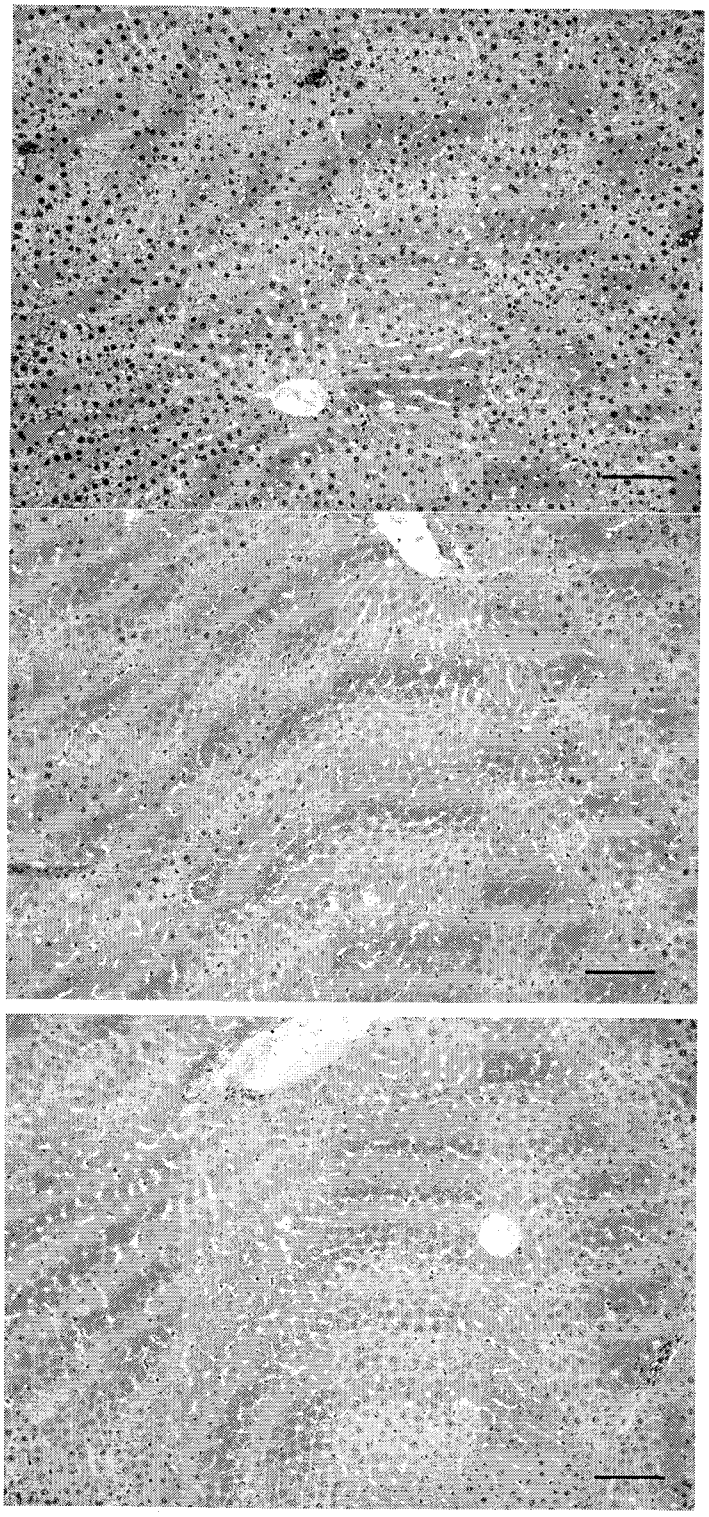


Figure 3.5: Photomicrographs of haematoxylin stained liver from control (top), I3C (middle), and DIM (bottom) treated male rats at 12 month time point. The preparation of tissues was as described in the Materials and Methods. Bar= 50 μm.

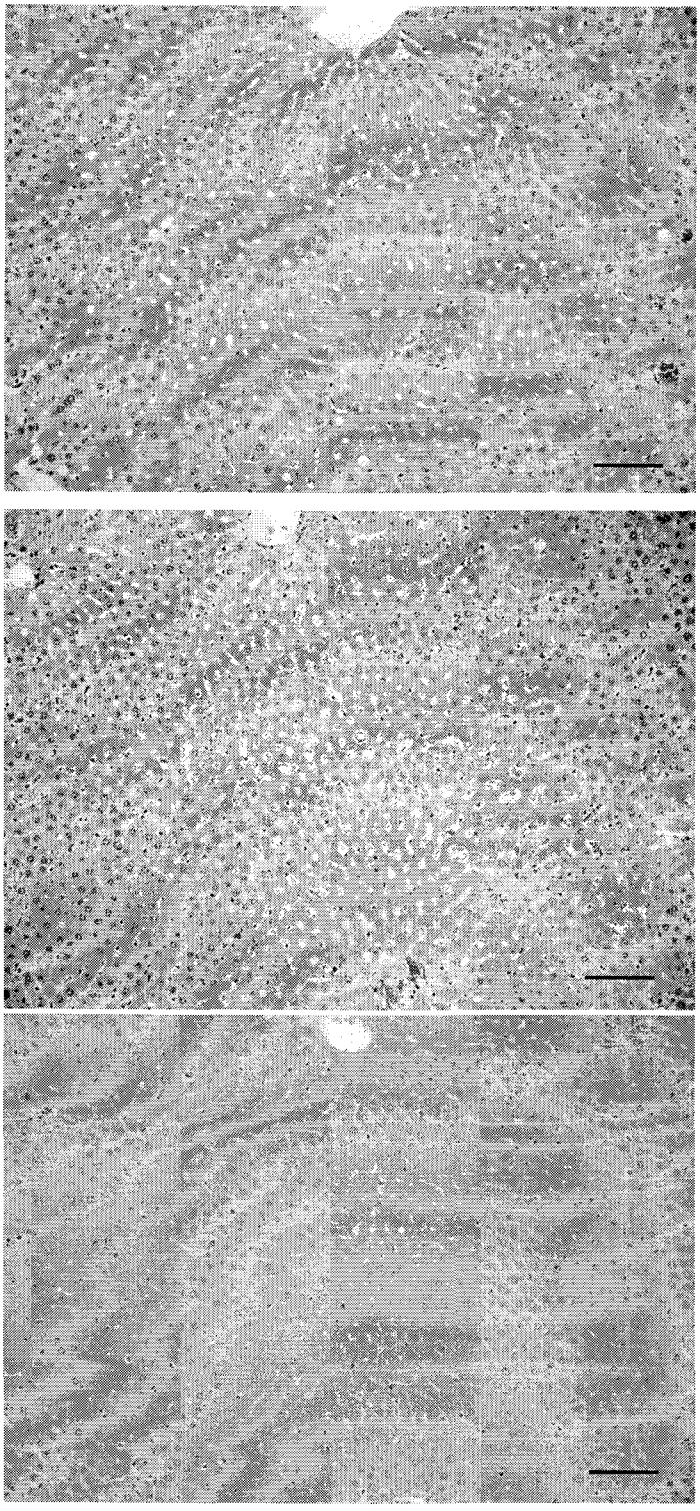


Figure 3.6: Photomicrographs of haematoxylin stained liver from control (top), I3C (middle), and DIM (bottom) treated female rats at 12 month time point. The preparation of tissues was as described in the Materials and Methods. Bar= 50 μm.

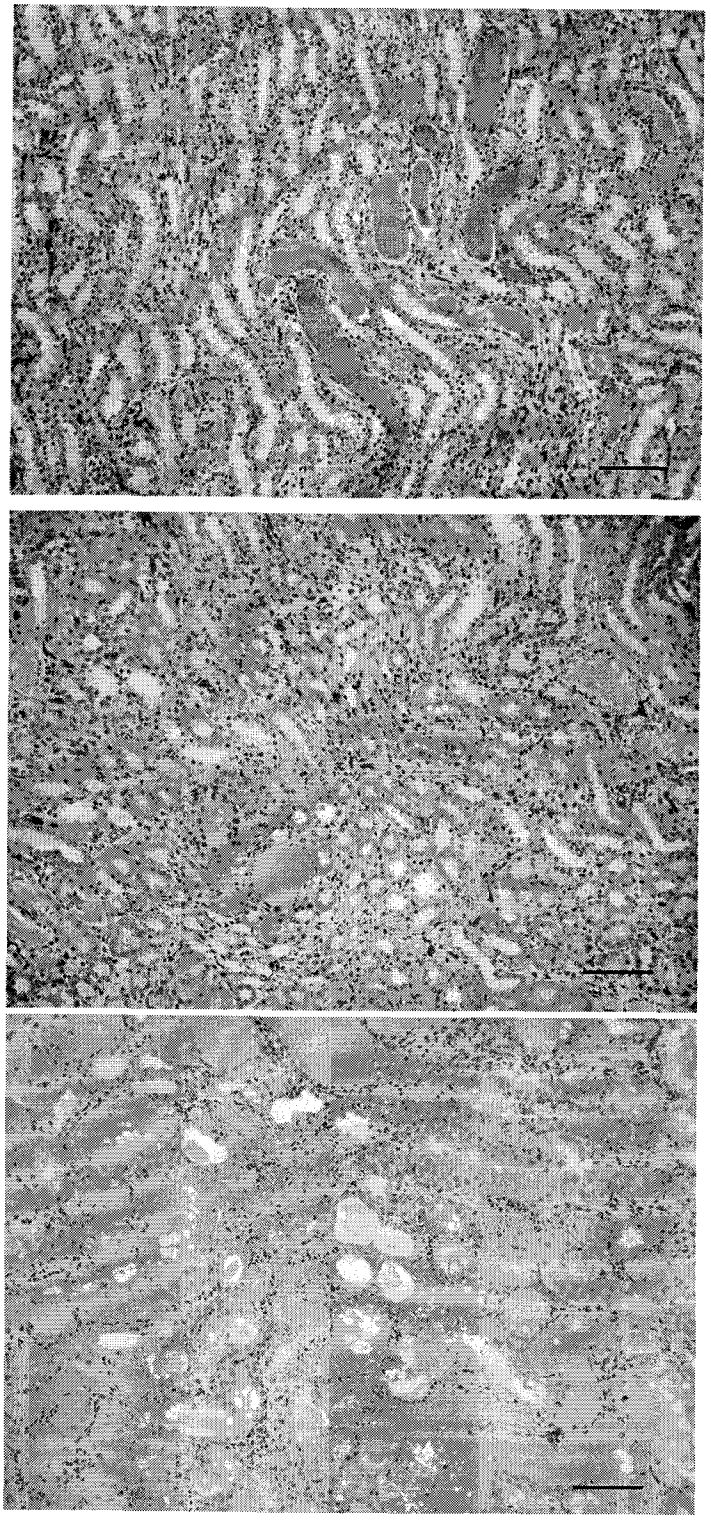


Figure 3.7: Photomicrographs of haematoxylin stained kidney from control (top), I3C (middle), and DIM (bottom) treated male rats at 12 month time point. The preparation of tissues was as described in the Materials and Methods. Bar= 50 μm.

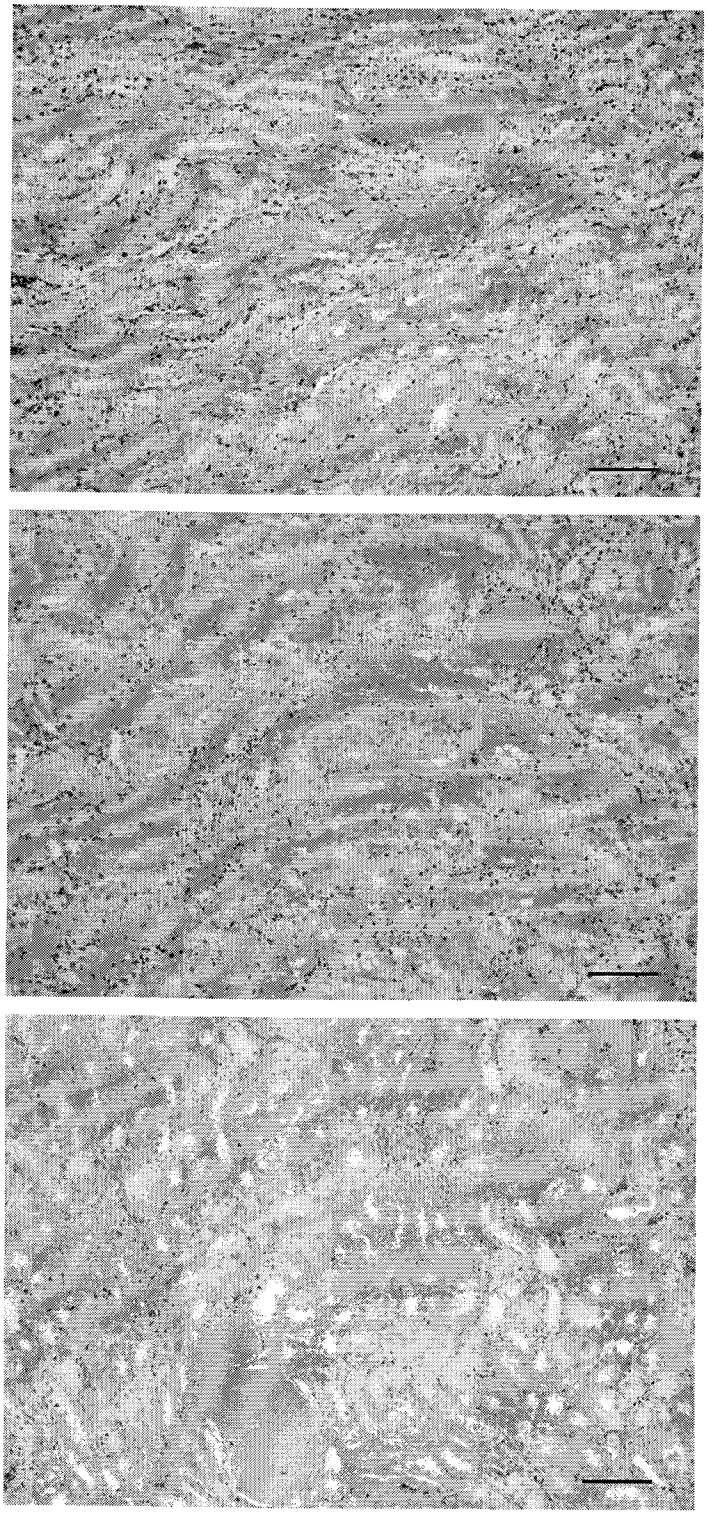


Figure 3.8: Photomicrographs of haematoxylin stained kidney from control (top), I3C (middle), and DIM (bottom) treated female rats at 12 month time point. The preparation of tissues was as described in the Materials and Methods. Bar= 50 μm.

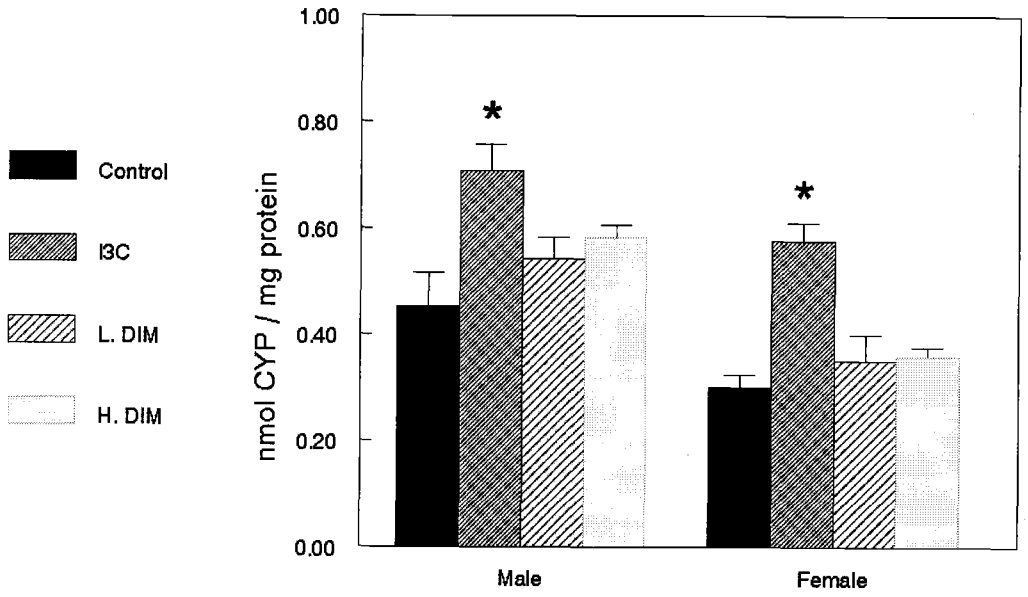


Figure 3.9: Total hepatic microsomal CYP levels in male and female rats after 12 months of dietary exposure. The bars represent group averages ± SE. * = $p<0.05$.

induced CYP1A1/1A2 but to a much lesser degree (2- and 10-fold for males and females, respectively, data not shown). Immunoquantitation of CYP1A1 and CYP1A2 in liver of rats at 12 months is shown in figure 3.10 (males) and figure 3.11 (females). Relative band densities of hepatic CYP1A1 were increased 82 ($p<0.001$)- and 16 ($p=0.032$)-fold in male rats fed diets containing I3C or the high dose of DIM, respectively. CYP1A1 was not detected in the livers of control female rats but band densities were induced to levels 9-fold higher in I3C

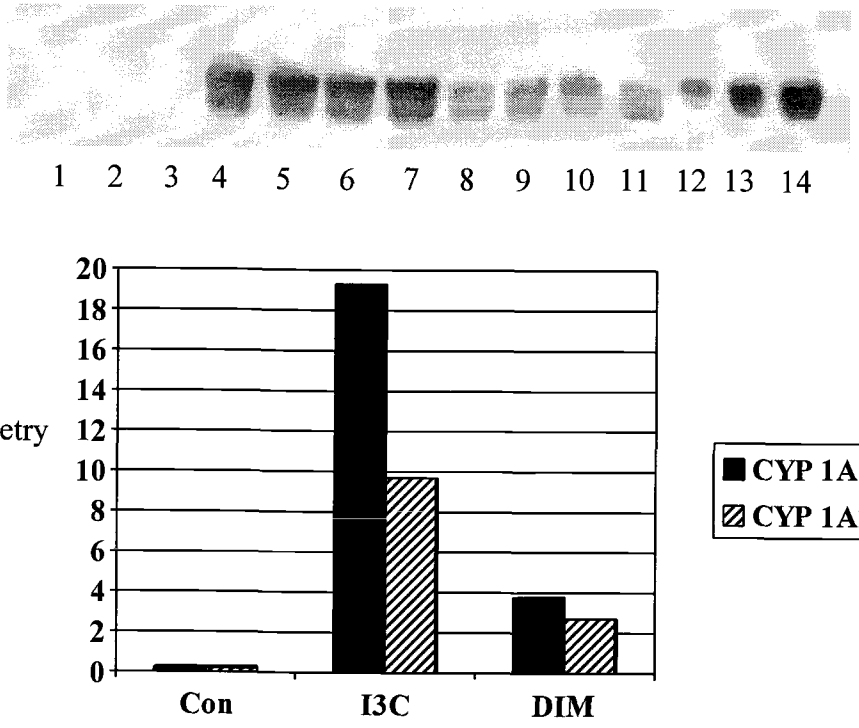
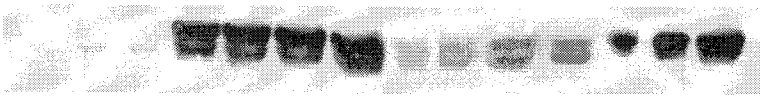


Figure 3.10: Western blot (inset) and densitometry of CYP 1A1 and 1A2 in liver micromsomes from male rats administered control diet, I3C or the high dose of DIM for 12 months. Microsomal proteins (24 μg) from rat livers were resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed with a goat antibody that recognizes both rat CYP 1A1 and 1A2. Lanes 1-3 were controls; lanes 4-7 rats fed I3C; lanes 8-11 rats fed the high dose of DIM; lanes 12-14 were CYP1A1 standards (0.5, 1.0 and 2.0 pmol).

supplemented animals than in the DIM treated group. The band densities of hepatic CYP1A2 were elevated approximately 40-fold ($p<0.001$) in both male and female rats given I3C but only 10-fold in rats fed diet containing the high dose of DIM. In the colon, CYP1A1 band densities were induced 10- and 8-fold (males) and 8- and 3-fold (females) in the I3C and DIM groups, respectively (Figure 3.12). No evidence was seen for CYP1B1 induction in the colon of either sex following



1 2 3 4 5 6 7 8 9 10 11 12 13 14

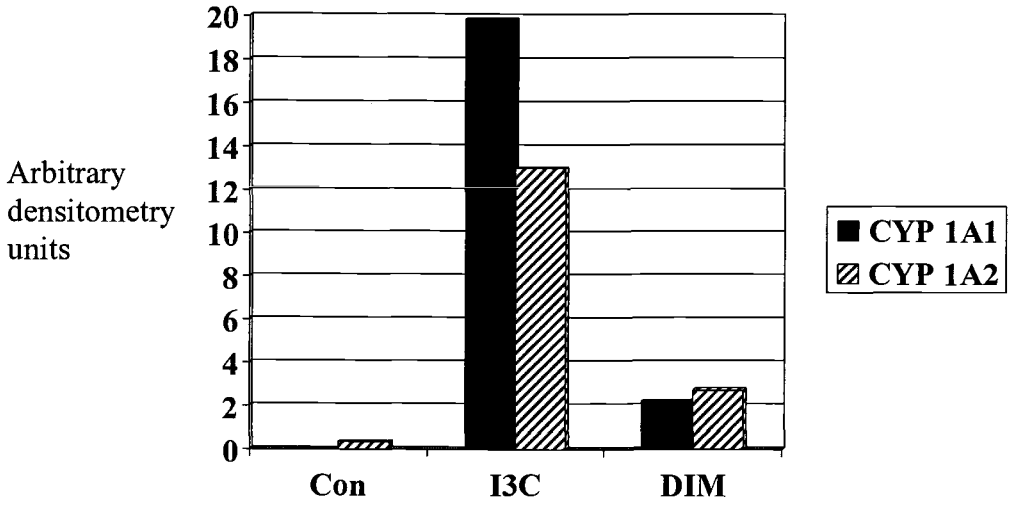


Figure 3.11: Western blot (inset) and densitometry of CYP 1A1 and 1A2 in liver micromsomes from female rats administered control diet, I3C or the high dose of DIM for 12 months. Microsomal proteins (24 μg) from rat livers were resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed with a goat antibody that recognizes both rat CYP 1A1 and 1A2. Lanes 1-3 were controls; lanes 4-7 rats fed I3C; lanes 8-11 rats fed the high dose of DIM; lanes 12-14 were CYP1A1 standards (0.5, 1.0 and 2.0 pmol).

administration of I3C or DIM for 12 months (data not shown). After 12 months, in females exposed to I3C, CYP3A2 band densities were increased 5- and 2-fold with I3C and DIM, respectively (Figure 3.13). I3C induced the CYP 3A2 band density almost 2-fold in male rats but no induction was observed with DIM exposure.

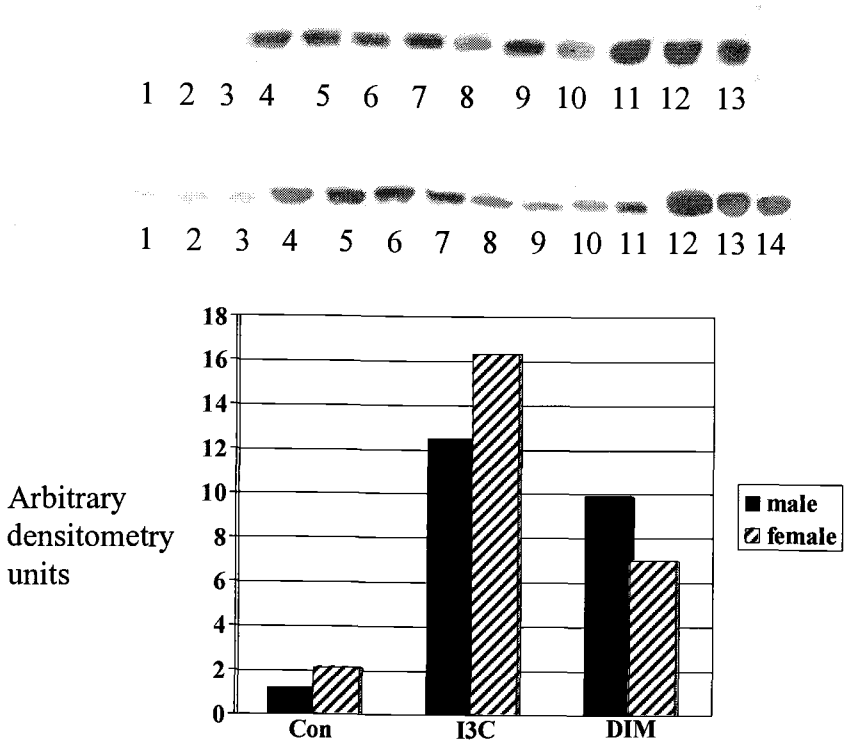


Figure 3.12: Western blot (inset) and densitometry of CYP1A1 in colonic lysates from male and female rats administered control diet, I3C or the high dose of DIM for 12 months. Lysate proteins (80 μg) from rat colon were resolved by SDS-Page, blotted onto nitrocellulose and probed with goat antibody to rat CYP1A1 as described in Materials and Methods. Lanes 1-3 are from rats fed control diet; lanes 4-7 from rats fed I3C; lanes 8-10 (male, top inset) and 8-11 (female, bottom inset) from rats fed the high dose of DIM and lanes 11-13 (top inset) or 12-14 (bottom inset) were 1.0, 0.5 and 0.25 pmol, respectively of CYP1A1 standard.

Bone density

Qualitative and histomorphometric analysis of methacrylate-embedded undecalcified cancellous and cortical bone from mature Sprague-Dawley rats showed that, in comparison to the control group, the I3C and DIM diets were not associated with any additional adverse effects on bone density, structure, or turnover. All three groups had cancellous osteoporosis more severe than that

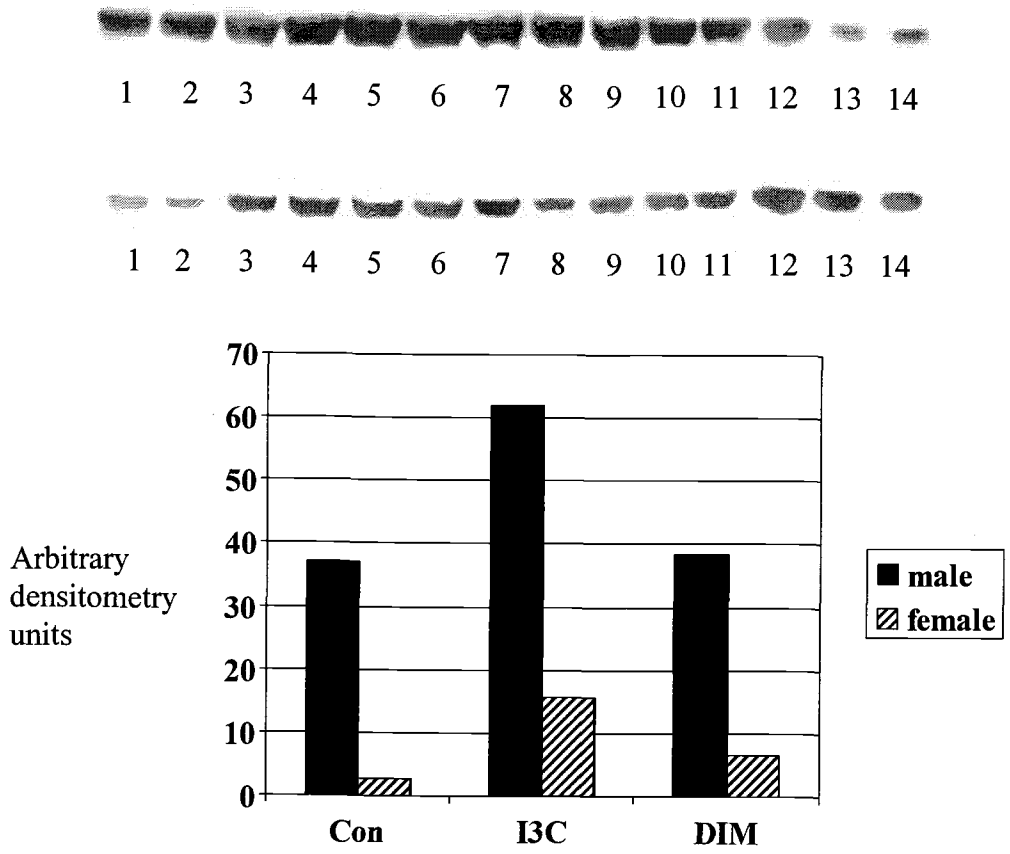


Figure 3.13: Western blot (inset) and densitometry of CYP3A2 in liver microsomes from male (top inset) and female (bottom inset) rats administered control diet, I3C or the high dose of DIM for 12 months. Microsomal proteins (24 μg) were resolved by SDS-PAGE, blotting onto nitrocellulose and probed with goat antibody to rat CYP3A2 as described in Materials and Methods. Lanes 1-3 (male) and 1-2 (female) are from rats fed control diet; lanes 4-7 (male) and 3-7 (female) are from rats fed I3C diet; lanes 8-11 are from rats fed the high dose of DIM; and lanes 12-14 are 1.0, 0.5 and 0.25 pmol, respectively of purified CYP3A2.

observed in control rats from previous studies where other diets were utilized. This osteoporosis was more severe in male than female rats (data not shown).

Discussion

The doses tested in the study were based on the current maximal human dose of DIM of 2 mg/Kg which was provided by a daily dose of 6.6 mg/Kg formula weight of BioResponse-DIM® (Indoloplex®). Twenty mg/Kg DIM was provided by a daily dose of 66 mg/Kg BioResponse-DIM™ to give an exposure 10 times higher than the current human dose, to amplify any potential toxicity not apparent at the lower dose. The I3C exposure utilized (50 mg/kg/day) a dose shown in previous studies to result in levels of DIM in human blood complimentary to the level observed when exposed to 66 mg/Kg/day BioResponse-DIM® (Arneson *et al.*, 2001; Zeligs *et al.*, 2002) and that represents approximately 5-7 times the daily dose recommended by commercial suppliers of I3C supplements.

The body weights and organ weights did not suggest any chronic treatment-related toxicity with the possible exception of liver. A previous study showed that after a 15 day treatment of 0.5% I3C in the diet of rats, the liver somatic index was significantly increased from 4.4% in controls to 6.4% (Manson *et al.*, 1997). The smaller but chronic doses of I3C and DIM in this study also resulted in a significant increase in the LSI. A 3 month oral administration of I3C to rats at doses of 4, 20 or 100 mg/Kg/day also demonstrated an increased LSI (at the 20 and 100 mg/Kg/day doses). In that same study, histopathological changes in liver were observed at the 100 mg/Kg/day dose as well as a decrease in testes weights at all doses (NCI, 1996). These differences may be related to the method of administration (diet versus gavage). This increase in LSI observed in our study

with I3C or DIM correlates with the degree of CYP induction that was also observed, as I3C was a more efficacious inducer of CYP and also had a greater effect on LSI when compared to DIM. I3C significantly enhanced LSI in males after 3 or 12 months. The high dose of DIM also increased LSI but only after 12 months. The increase was not as marked as with I3C and was seen only in males. We have no explanation for the effect of sex on LSI.

Clinical chemistry panels failed to uncover any significant differences between control, I3C, and DIM treated rats that would indicate toxicity. Conversely, the significant reduction in creatine kinase (CK) in male rats fed DIM, and the significant reduction in alkaline phosphatase (ALP) and aspartate aminotransferase (AST) in rats fed I3C or DIM could indicate possible protective effects against age related tissue damage. This may be explained by the antioxidant and electrophilic scavenging properties described for I3C and DIM (Arnao *et al.*, 1996; Fong *et al.*, 1990; Shertzer *et al.*, 1988; Shertzer and Senft, 2000). Again, this significant reduction in the serum enzyme markers were only evident in male rats after 12 months of dietary exposures. With one exception, no other significant alterations in serum chemistry following 12 months of exposure to I3C or DIM was evident. In previous work some acid condensation products of I3C were shown to lower serum LDL/VLDL cholesterol levels in mice (Dunn and LeBlanc, 1994), resulting from the inhibition of acyl-CoA:cholesterol acyltransferase (ACAT). Treatment with I3C or DIM failed to provide cholesterol lowering effects in this study. The dietary indoles did not alter serum levels of testosterone in males or E_2 in females. The

fact that there were no significant changes in testosterone levels in this study may not be surprising considering the individual variation normally observed in testosterone levels in rats (Overpeck *et al.*, 1978) and that 750 mg/kg of I3C were needed to cause a significant effect in shorter-term studies (Wilson *et al.*, 1999). Clinical trials with I3C have documented reductions in urinary E_2 levels in both men and women concurrent with an increase in the 2-OH-E_2/16α-OH-E_2 ratio (Michnovicz *et al.*, 1997) and absorption-enhanced DIM also increased this ratio in a pilot clinical study (Zeligs *et al.*, 2002).

The basis for investigation of both the 25-OH-D_3 levels and bone density stem from reports of individuals with low 25-OH-D_3 and a decrease in bone density while on I3C. The concern regarding vitamin D_3 is that enzyme induction in the colon or liver could influence levels of this vitamin/hormone which in turn could affect bone density. It has also been shown that bone density can be influenced by estrogen metabolism, especially CYP1A-dependent 2-hydroxylation (Leelawattana et al., 2000). There appears to be greater bone density with lower ratios of 2-OH/ 16α-OH estrogen levels in postmenopausal women, implying that increasing this ratio with I3C or DIM could result in lower bone density. No effect on bone density was observed in this study following I3C or DIM exposure. DIM had no significant effects on 25-OH-D_3 levels, however significant increases were observed in males fed I3C for 3 or 12 months and in females fed I3C for 12 months.

The absence of data indicating toxicity in the chemistry panel and other blood work was confirmed by the histopathological examination. Other than the increase

in hyaline casts in the kidney, no apparent lesions were observed in any of the tissues examined. The appearance and severity of this kidney pathology increased with age but was not treatment related.

The induction of CYP isoforms observed in this study is mostly consistent with data from previous acute or sub-chronic studies. The 24-, 3-, and 4- fold induction of CYPs 1A1, 1A2, and 3A1/2, respectively, in male Fischer 344 rats consuming 0.2% I3C in their diet for seven days (Stresser *et al.* 1994) can be compared to the 82-, 40-, and 2- fold inductions observed in male Sprague-Dawley rats in this study after administration of a similar dose of I3C over a much longer time.

Whereas direct toxicity by long-term exposure to I3C and DIM is not evident in this study, the induction of CYPs, especially those of the 1A subfamily, could be a cause for concern, given the role of these enzymes in activation of polycyclic aromatic hydrocarbons such as benzo[a]pyrene and aromatic amines such as 4-aminobiphenyl or PhIP. The induction of CYP 3A may also be significant as this subfamily contributes to the metabolism of 50-60% of all clinically relevant drugs (Guengrich, 1999). The dampened induction of CYPs seen with DIM exposure may result in fewer drug interactions with DIM supplementation, when compared to I3C. When all endpoints in this study are considered in the comparison between I3C and DIM, the differences can be contributed to a magnified effect in the increased liver somatic index, total CYP, and induction of specific CYPs in the I3C treated group. The higher efficacy/potency of I3C is expected and related to the fact that in the acidic conditions of the stomach after oral exposure, I3C becomes a

complex mixture of not only DIM but more than 20 different I3C-derived compounds, all having pharmacological/toxicological effects, such as possessing different affinities for the Ah receptor. One of these compounds, ICZ, binds to the Ah receptor with an affinity similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). DIM has been shown to be relatively more stable in acid and does not robustly undergo further condensation reactions.

The data from this study confirm results from short-term studies indicating that both I3C and DIM are relatively non-toxic compounds. Furthermore, these results confirm earlier long-term feeding studies in other models, including the rainbow trout and the same strain of rat used in the present study, that I3C is not a complete carcinogen (Dashwood *et al.*, 1991; Oganesian *et al.*, 1999; Stoner *et al.*, 2002). A concern, however, with the prolonged use of I3C for cancer chemoprevention is its potential for promotion of liver neoplasms. I3C is an effective promoter of liver cancer in the trout (Dashwood *et al.*, 1991; Oganesian *et al.*, 1999) and recent studies in a multi-organ model (female Sprague-Dawley rats initiated with 7,12-dimethylbenz[a]anthracene for breast, aflatoxin B_1 for liver and azoxymethane for colon) demonstrate that long-term post-initiation with dietary I3C could provide some chemoprotection in breast and colon, but not without a significantly increased risk for liver neoplasms (Stoner *et al.*, 2002). The long-term post-initiation effects of I3C in hepatocarcinogenesis are not consistent across species as this treatment with C57 black mice, initiated at 15 days of age with diethylnitrosamine, provided significant protection (Oganesian *et al.*, 1997). The mechanism(s) of I3C tumor

modulation need to be established in these models in order to assess the risk to human health.

Previous work from our laboratory utilizing the rainbow trout has shown both I3C and DIM to be estrogenic (Shilling & Williams, 2000; Shilling *et al.*, 2001). The estrogenicity of I3C is a likely mechanism by which I3C promotes hepatocarcinogenesis in trout (Oganesian *et al.*, 1999). We have not yet tested DIM in trout as a tumor promoter. DIM is primarily an anti-estrogen in mammalian systems (Chen *et al.*, 1998; McDougal *et al.*, 2001). We hypothesize that this difference may be a function of species-specific DIM metabolism. We have preliminary evidence that CYP-dependent hydroxylation of DIM is required in trout to elicit estrogenicity (Shilling *et al.*, 2001).

Also confirmed were the alterations observed in the monooxygenase system that could be important in carcinogen bioactivaion/detoxication and potential adverse effects on drug metabolism. The results from this study suggest that DIM is a markedly less efficacious inducer of CYP in the rat but further studies are required to investigate the effects of both I3C and DIM on carcinogenesis, metabolism, and human health.

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CHAPTER 4

Investigation of Indole-3-Carbinol and 3,3'-Diindolylmethane Induced Alterations in Drug Metabolism Utilizing High-Precision Rat Liver Slices

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Abstract

Indole-3-carbinol (I3C) and 3,3'-diindolylmethane (DIM) are naturally occurring dietary components found in cruciferous vegetables. They are now marketed as dietary supplements and are under investigation as chemopreventive agents, despite limited data on the effects of chronic exposure. Among the concerns for chronic exposure is the well documented potential for phytochemicals to trigger drug interactions through modulation of enzyme levels and activities. This concern is relevant in the case of I3C and DIM, as it has been demonstrated that short-term exposures of rats to I3C and DIM are capable of altering monooxygenase levels. I3C and DIM repress expression of flavin-containing monooxygenase (FMO) 1 in liver and intestine of rat with concurrent induction of several isozymes of cytochrome P450 (CYP). FMO and CYP have overlapping substrate specificities, but usually yield different products. I3C and DIM can thereby alter the metabolic profile of drugs and xenobiotics. The current study utilizes the high precision liver slice model to investigate the potential for chronic exposures to I3C and DIM to alter drug metabolism. Rats were fed either control diet, 5-7-X the maximal recommended dose of I3C or 10-X the current human dose of absorption-enhanced DIM for 12 months. Slices were prepared from liver and incubated with ^{14}C- N,N-dimethylaniline (DMA) for 30 minutes. DMA is a substrate for both FMO (N-oxygenation) and CYP (N-demethylation). A high performance liquid chromatography (HPLC) assay was employed to quantitate ^{14}C-

DMA metabolites. Incubations of ^{14}C- DMA with slices from control rats resulted in approximately 90% of the total metabolites formed as the result of FMO mediated N-oxygenation and approximately 10 % the result of N-demethylation by CYP. The percentage of metabolites formed from each pathway was around 50% in rats treated with I3C. The metabolic contribution of FMO and CYP in incubations with slices from DIM treated rats was 80% and 20%, respectively. The modulation of DMA metabolism observed in this study confirms our concern that chronic I3C and DIM exposure may result in significant alterations to the efficacy and toxicity of drugs and/or xenobiotics. Further studies should be performed to determine the potential for a similar response in humans.

Abbreviations: I3C, indole-3-carbinol; DIM, 3,3'-diindolylmethane; DMA, N,N-dimethylaniline; CYP, cytochrome P450; FMO, flavin-containing monooxygenase; ACPs, acid-condensation products.

Introduction

Indole-3-carbinol (I3C) is a naturally occurring plant alkaloid formed by the hydrolysis of indole glucosinolate (glucobrassicin), found in significant concentration in cruciferous vegetables such as broccoli and Brussels sprouts (Bradfield and Bjeldanes, 1987; Slominski and Campbell, 1987; McDanell *et al.*, 1988; Preobrazhenskaya *et al.*, 1993). Furthermore, I3C is an unstable compound that undergoes rapid oligomerization in the acid environment of the stomach to form dimers, trimers, tetramers and several other higher order condensation products (Leete and Marion, 1953; Bjeldanes *et al.*, 1991; Wortelboer *et al.,* 1992). The major product formed *in vitro* (Spande, 1979) and *in vivo* after oral administration (Dashwood *et al.,* 1989; Stresser *et al.*, 1995) is 3,3-diindolylmethane (DIM).

Both I3C and DIM are marketed as dietary supplements and are under investigation as potential chemopreventive agents, despite the fact that little is known about the effects of chronic exposure. One area of concern for chronic exposure to such phytochemicals is the well documented occurrence of drug-drug interactions as a result of phytochemical induced modulation of metabolizing enzymes. The most prominent example involves alteration of blood levels of drugs in patients consuming grapefruit juice (Bailey *et al.*, 1994). In this case furanocoumarins in the grapefruit juice act as mechanism based inhibitors and

inhibit the expression and activity of CYP3A4, a CYP isoform responsible for the metabolism of 50-60% of all therapeutic drugs (Guengrich, 1999). This results in an improved bioavailability of drugs such as midazolam, cyclosporine, and felodipine leading to an increase in efficacy or enhanced toxicity depending on the therapeutic index.

The concerns over potential drug-drug or food-drug interactions as a consequence of I3C and DIM exposure are substantiated by data from previous studies demonstrating similar enzyme interactions. The induction of numerous CYP isoforms resulting from dietary exposure of I3C and DIM in rats has been well documented. Short term I3C exposure produced 2-4 fold inductions of CYP1A2, CYP2B1/2, and CYP3A with over 20-fold increases observed in CYP1A1 (Stresser *et al.*, 1994). Chronic exposures, as studied in the current experiment, resulted in even stronger 40- and 30- fold inductions for CYP1A1 and CYP3A2, respectively (Leibelt *et al.*, 2003a; Leibelt *et al.*, 2003b). Sub-chronic exposures to I3C have also been shown to inhibit FMO expression and activity in the rat in a time- and dose-dependent fashion (Larsen-Su and Williams, 1996). For drugs metabolically inactivated by FMO a trend of increasing efficacy or toxicity could occur with increasing exposure to I3C or DIM, whereas the opposite could occur with CYP induction as an increase in metabolism could lead to diminished potency.

The potential effects that the combined CYP induction and FMO inhibition have on drug metabolism were documented by Katchamart *et al.* (2000). This

study demonstrated that the ratio of FMO/CYP mediated metabolism of N,N-dimethylaniline, nicotine, and tamoxifen in incubations with liver microsomes decreased after rats were administered 2500 ppm DIM in their diets for 4 weeks. This decrease in metabolite ratio was attributed to an inhibition of hepatic flavin-containing monooxygenase isoform 1 (FMO1)-dependent formation of the N-oxide with a concurrent induction of CYP-mediated N-demethylation.

Precision-cut liver slices have become widely accepted as a suitable *in vitro* tool to address questions regarding drug metabolism, and the induction properties of drugs and xenobiotics (Miller *et al.*, 1993; Lake *et al.*, 1996; Edwards *et al.*, 2003). Liver slices have the advantage over other *in vitro* systems in that the structural integrity of the organ, the cell heterogeneity and the cell-cell interactions are retained (Parrish *et al.,* 1995). Numerous studies have confirmed the reliability of slices in mimicking *in vivo* conditions and slices have been shown to up-regulate CYP expression in response to chemical treatments as observed in studies employing inducers such as β-naphthoflavone, phenobarbital, rifampcin, and I3C (Ekins *et al.*, 1995; Oganesian *et al.*, 1997; Renwick *et al.*, 1999; Edwards *et al.*, 2003). Radiolabelled ligand metabolism and fate studies have verified the ability of slices to metabolize compounds in a way consistent with *in vivo* metabolism (Price *et al.*, 1995; Ball *et al.,* 1996; Worboys *et al.*, 1996).

The aim of the current study was to investigate the effects that chronic exposure to I3C and DIM and subsequent enzyme modulation may have on

drug/xenobiotic metabolism utilizing the rat liver slice model. Considering results from previous studies, it would be expected that incubations of a xenobiotic, that is substrate for both CYP and FMO, with slices from rats exposed to I3C and DIM would result in altered metabolism in favor of higher CYP mediated metabolism.

Materials and Methods

Chemicals

Indole-3-carbinol (I3C) and [U-^{14}C] N,N-dimethylaniline (DMA) (15.5 μCi/μmol) were obtained from Sigma-Aldrich Co. (Milwaukee, WI). 3,3'-Diindolylmethane (BioResponse DIM™) was provided by BioResponse, LLC (Boulder, CO).

Animals

Six male Sprague-Dawley rats were purchased from Simonsens (Gilroy, CA) at four weeks of age. After a one week acclimation period, animals were randomly divided into 3 different treatment groups. Rats were housed individually in hanging metal wire cages at the Laboratory Animal Resource Center, Oregon State University and maintained at 22°C and 40 to 60 % humidity on a 12 hour light/dark cycle. Both tap water and powdered semisynthetic diet were available *ad libitum* throughout the study. Group 1 received control (AIN-76A) diet. The diet for groups 2 and 3 were supplemented with I3C or BioResponse DIM™ to levels providing a dose of 50 mg/Kg/day or 66 mg/Kg/day, respectively. Rats were weighed and food consumption estimated weekly for the first three months and monthly thereafter. At the end of one year all diets were removed overnight before sampling. Rats were anesthetized and then euthanized by exsanguination according to a protocol approved by the Oregon State University Institutional Animal Care and Use Committee.

Sample Collection and preparation

The liver was removed from each animal and stored in ice cold Waymouth MB 752/1 medium (Sigma-Aldrich) supplemented with 10 mM HEPES, 8 mM sodium bicarbonate, 50 mg/ml gentamicin and 10% fetal calf serum. All media were filter-sterilized, using a 0.22 μm Gelman Vacucap filter (Fisher Scientific) into sterile containers and stored at 4°C. The same media were used for transportation of livers, slice preparation, and incubation. All glassware and equipment was sterilized at 105°C for 30 minutes prior to use.

High-precision liver slices

Using a stainless steel tissue coring press, 8 mm cores were generated from the largest lobe of each rat liver and then cut into precision slices (250 μm in thickness) using a Krumdieck tissue slicer (Alabama Research and Development Corp., Munford, AL). The slices were distributed into 12-well Falcon plates (one slice per well) in triplicate, containing 1 ml of the previously described Waymouth media per well. Additional slices were distributed to separate plates for use as controls and for the ATP assay described below. All plates were covered and preincubated for 1 hour inside a tight-seal container saturated with 95% O_2 / 5% CO_2. Containers were kept inside an incubator at 34°C on an orbital shaker set at 90 RPM.

DMA incubations

After 1 hour preincubation, slices were transfered to new plates containing 100 μM [^{14}C] -DMA and incubated for an additional 30 minutes. [^{14}C] -DMA was added to Waymouth media, prior to introduction of slices, using dimethylsulfoxide as a vehicle (0.5% of final volume) for a final volume of 1 ml per well. After 30 minutes both media and slice were removed from the well for extraction.

Extraction

An equal volume (1 ml) of cold methanol was added to media samples from each well and spun at 10,000 g in a table top centrifuge for 5 minutes at 4°C to precipitate protein. The supernatant was removed for HPLC analysis.

The triplicate slices from each rat were pooled and then homogenized in 500 μl phosphate buffer containing 30% glycerol, 1mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylflouride, pH 7.4. An equal volume (0.8 ml) of cold methanol was added and the samples spun at 10,000 g in a table top centrifuge for 5 minutes at 4°C. The supernatent was removed for HPLC analysis.

HPLC analysis

FMO and CYP activity toward [^{14}C] -DMA was determined utilizing an HPLC assay to simultaneously measure metabolites formed by either FMO-mediated N-oxygenation or CYP- mediated N-demethylation (Williams, 1991; Shehin-Johnson *et al.*, 1995) (Figure 4.1). Samples from media and slice homogenates were transferred to autosampler vials and placed in the sample

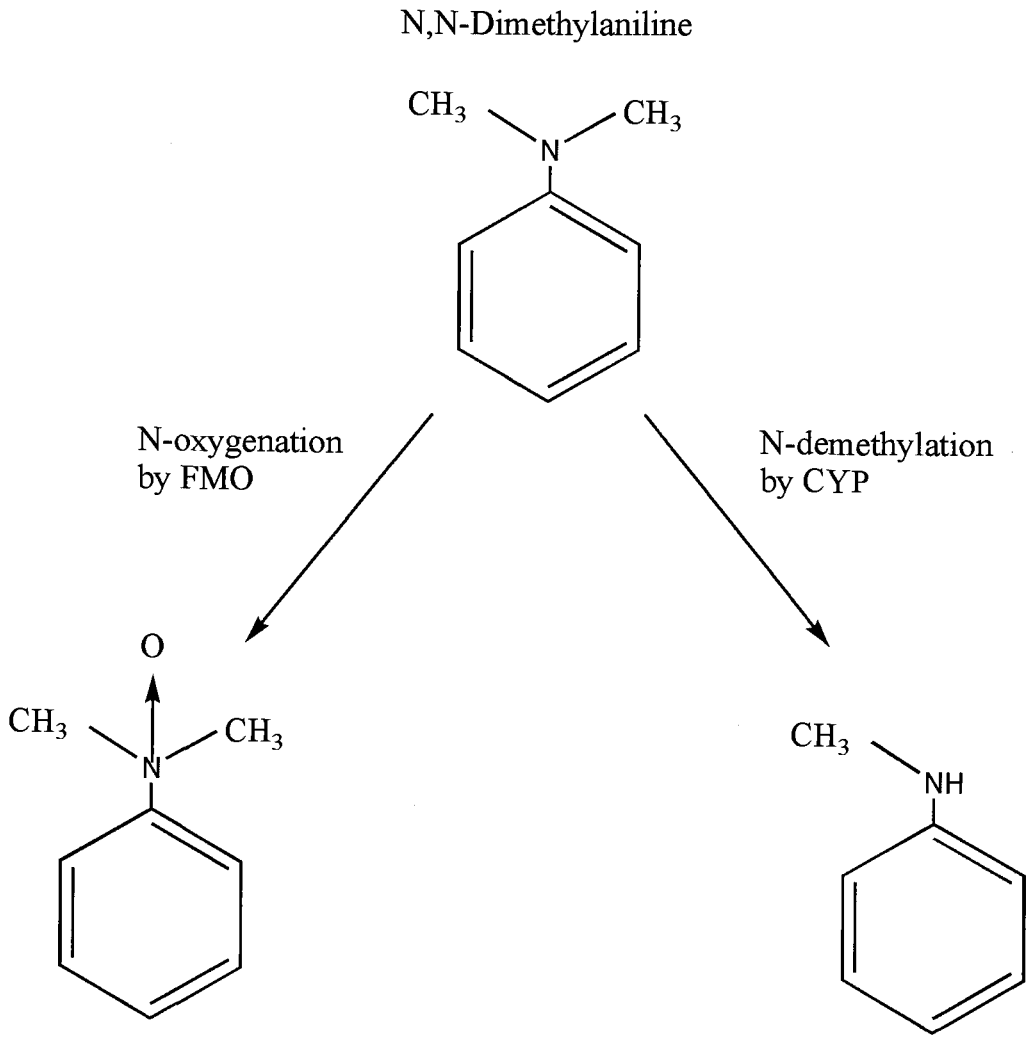

Figure 4.1: Metabolism of N,N-dimethylaniline. N-oxygenation by flavin-containing monooxygenase (FMO) and N-demethylation by cytochrome P450 (CYP).

compartment (maintained at 10°C) of the Waters 2690 HPLC (Milford, MA). Chromatography of the samples was performed using a 4.6 mm X 10 cm Whatman RAC II Partisil 5-ODS 3 column (Fisher Scientific) in a 35°C, temperature-

controlled compartment. Elution was performed using 62% methanol: 38% water with a constant flow rate of 0.5 ml/minute. Both an inline Packard TR505 radiometric detector (0.5 ml cell, 1.5 ml/min scintillant) and a Waters 996 Photodiode Array detector were used for detection. Relative retention times of DMA (7-8 min.), methylaniline (4-5 min.), and DMA N-oxide (2-3 min.) are demonstrated in a representative chromatogram trace (Figure 4.2).

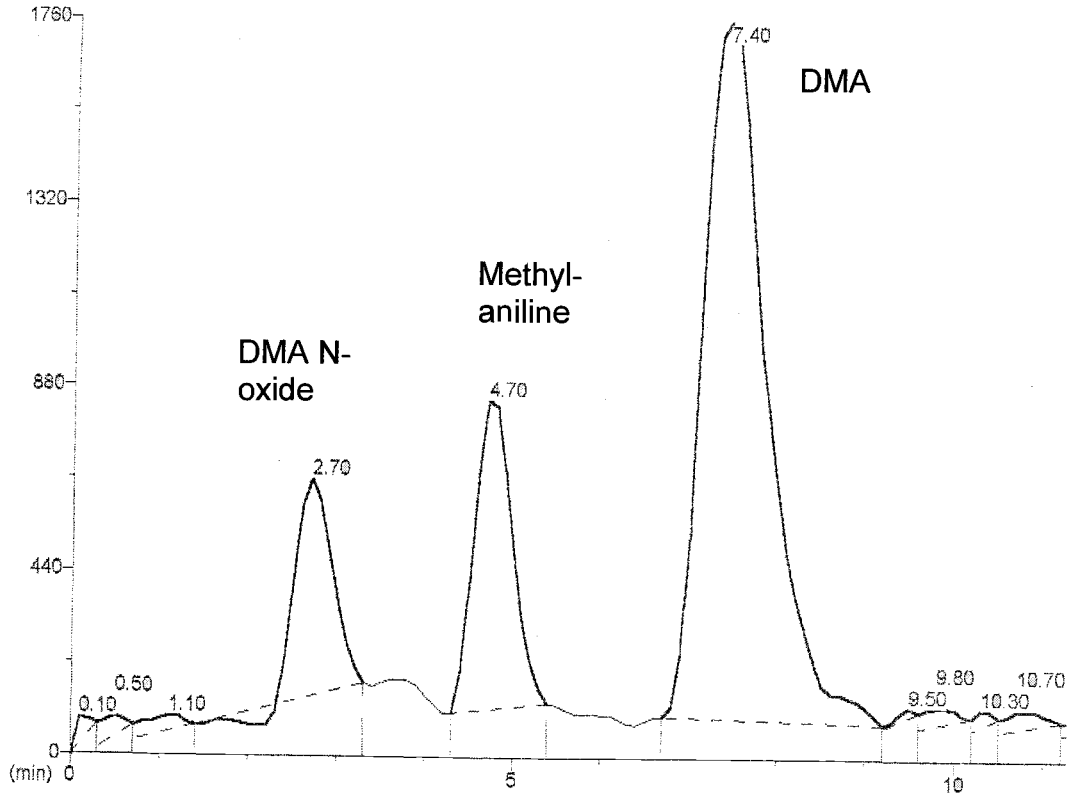

Figure 4.2: HPLC chromatogram demonstrating separation of DMA (retention time=7.4 minutes), methylaniline (4.7 minutes) and DMA N-oxide (2.7 minutes).

ATP assay

At time 0 and after final incubation, ATP production was used as a marker to test for cell viability and signs of cytotoxicity. ATP production was measured using kits purchased from Sigma Chemicals based on a procedure described by Adam (1963).

Results

The viability of slices was assessed by analyzing ATP levels in slice homogenates before treatment and after incubation with DMA. No signs of toxicity were observed as post-incubation ATP levels were higher than in fresh time=0 control slices (Figure 4.3).

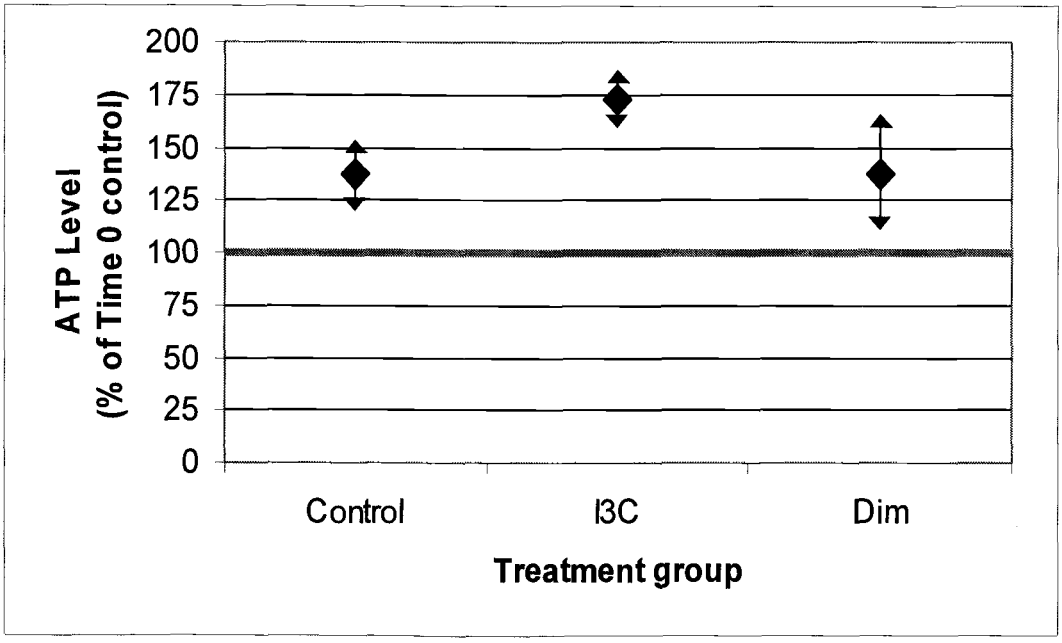

Figure 4.3: ATP levels in slice homogenates as a measure of viability after incubation with DMA. Expressed as % of ATP level found in fresh control slices. Values represent group averages ± SE.

The effects of chronic I3C and DIM treatment on CYP and FMO enzyme levels were assessed indirectly by quantitation of metabolites formed during the incubation with DMA of slices from rats exposed to I3C and DIM. Due to the lack of metabolites present in media, only homogenate samples were considered in

calculations of total metabolite percentages (Figure 4.4). When slices from control rats were incubated with DMA approximately 90 % of the total metabolites formed as a result of FMO mediated N-oxygenation and approximately 10 % of the total metabolites resulted from N-demethylation by CYP. In incubations with slices from I3C treated rats, the percentage of metabolites formed from each pathway was approximately 50 %. Slices from DIM treated rats produced levels of metabolites intermediate to those seen in the control and I3C treated animals with 80 and 20 % of the total metabolites resulting from N-oxygenation and N-demethylation, respectively.

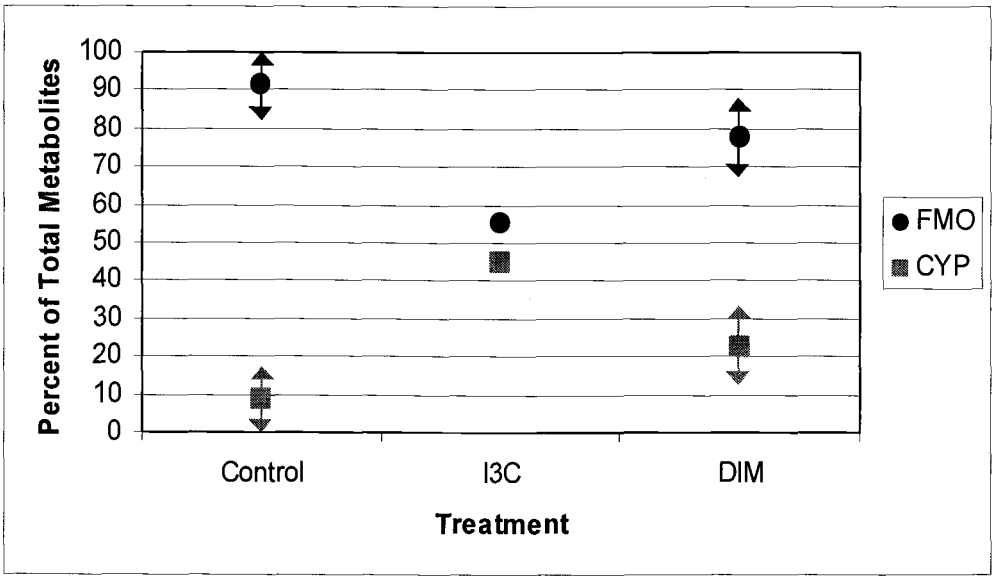

Figure 4.4: Percentage of total DMA metabolites produced from FMO N-oxygenation and CYP N-demethylation after incubation with liver slices from rats fed control diet or diet containing I3C or DIM for 1 year. Values represent group averages ± SE.

Discussion

Confirmation of cell viability is necessary in slice experiments to determine the extent of structural or biochemical damage that may introduce confounding variables to the model system. Histological assessment of slice viability was not performed in this study, as previous studies have shown the ATP assay to accurately predict the percentage of living cells (Oganesian *et al.*, 1997; Shilling and Williams, 2000). The post-incubation increase in cellular ATP levels in slices from all treatment groups confirms slice viability and indicates that the metabolic differences observed in this study can be attributed to enzyme activity and not toxicity. A post-incubation increase in ATP levels is also consistent with previous slice studies where it was observed that tissue slices take time to fully recover from processing.

Based upon the results of previous studies, we postulated that chronic dietary exposure to I3C and DIM could significantly alter the metabolic profile of drugs that are substrates for both monooxygenase systems. While not a pharmaceutical, DMA was a good candidate for our model system because of its previously characterized co-metabolism by CYP and FMO (Katchamart *et al.*, 2000). In this study most of the contribution for a shift in DMA metabolism appeared to come from an increase in CYP metabolism as opposed to a decrease in FMO metabolism, and DIM appeared to be less potent at altering the metabolic profile. This is consistent with unpublished data from our lab indicating that the

inhibition of FMO by I3C observed in short-term studies in the rat does not continue with chronic exposure, and in fact after 6 and 12 months of exposure to I3C the FMO levels return to that of untreated rats. The results are also consistent with a previous study demonstrating that CYP levels are highly inducible after chronic exposure to I3C, whereas DIM appeared to be a less efficacious inducer (Leibelt *et al.,* 2003). Independent of whether the change in metabolism came from a decrease in FMO mediated metabolism or an increase in CYP contribution, the marked shift in metabolism may result in significant changes to the toxicological and therapeutic properties of drugs.

Whether these findings can be extrapolated to humans depends on the similarities for regulation of these monooxygenase systems between the two species. While a comparable induction pathway of CYP in humans and rats mediated by the AHR is well documented, FMO responses are less well known. The major FMO in adult humans is FMO3 rather than FMO1. Human FMO1 is predominantly expressed in fetal liver and adult kidney. Previous studies have demonstrated a decrease in FMO3 activity towards trimethylamine in humans after being fed 300 g/day of Brussels sprouts, providing 0.002-0.014 mmol I3C/Kg/day (Cashman *et al.*, 1999). However, Brussels sprouts also contain numerous other phytochemicals like isothiocyanates and dithiolanes that may affect FMO levels and activity.

While the focus of this study has been on the effects of chronic exposure to I3C and DIM on drug metabolism, the observed shift in metabolism observed could also be important in carcinogen bioactivation and detoxification. In general, FMOs produce fewer bioactivated products than CYP (Cashman, 1995). This is especially relevant for CYPs of the 1A subfamily, given the role of these enzymes in activation of polycyclic aromatic hydrocarbons such as benzo[a]pyrene, and aromatic amines such as 4-aminobiphenyl and PhIP.

In summary, we have demonstrated that chronic oral administration of I3C and DIM to rats markedly alters the *in vitro* metabolism of DMA, a substrate for both CYP and FMO monooxygenases. It appears that the majority of this metabolic difference can be attributed to a strong induction of CYP that is consistent with I3C being a more efficacious inducer of CYP than DIM. The results of this study are important to human health as I3C and DIM are now marketed as supplements and proposed as chemopreventive agents for estrogen-dependent cancers. These compounds could be capable of causing metabolic alterations in humans similar to those observed in rats in this study, leading to effects on therapeutic efficacy or toxicity. Further studies need to be completed to determine the potential for a similar response in humans.

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CHAPTER 5

The Effects of Strain on I3C Tumor Modulation in the Mouse

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Abstract

Indole-3-carbinol (I3C), a naturally occurring dietary component found in cruciferous vegetables, has demonstrated chemoprotective properties in a number of animal studies. I3C is currently marketed as a dietary supplement and is under investigation as a chemopreventive agent against breast cancer, despite evidence that this compound is also capable of promoting hepatocarcinogenesis in some cancer models. In the current study, the infant mouse model was used to further elucidate whether I3C modulates carcinogenesis via estrogenic pathways. Infant male mice of a strain in which estrogens inhibit liver cancer (C57BL/6J, black) and a strain in which estrogens do not inhibit liver cancer (C57BR/cdJ, brown) were initiated with 5 mg/kg diethylnitrosamine (DEN), by i.p. injection, at 15 days of age. Nine days after initiation mice were weaned and immediately put on control diet or diet containing 1500 ppm I3C. After seven months, whole body and liver weights were recorded and the livers were sampled for tumors. A significant strain difference in sensitivity to DEN-initiated hepatocarcinogenesis was observed. I3C exposure resulted in a significant reduction in body weights for black mice initiated with DEN. I3C exposure did not influence body weight in brown mice but a significant increase in liver somatic index due to large tumor volumes was observed in brown mice initiated with DEN. I3C treatment did not result in significant tumor modulation in brown mice and with the exception of two mice that had unusually high tumor burdens (50-60x the next highest value), I3C treatment resulted in

approximately a 3-fold reduction in tumor multiplicity and volume in black mice. The results of this study suggest an estrogenic mechanism of tumor modulation by I3C in mice.

Introduction

Indole-3-carbinol (I3C) is a naturally occurring plant alkaloid formed from the hydrolysis of indole glucosinolate (glucobrassicin), found in significant concentrations in cruciferous vegetables such as broccoli, cauliflower, and Brussels sprouts (Slominski and Campbell, 1987; McDannell *et al.*, 1988). Glucobrassicin is hydrolyzed to glucose, sulfate, thiocyanate and I3C upon maceration of plant tissue at neutral pH, in the presence of the enzyme myrosinase. In an acidic environment, such as the stomach after oral exposure, I3C undergoes rapid oligomerization to form condensation products (I3C-ACP's) including 3,3'-diindolylmethane (DIM), 2-(indol-3-ylmethyl)-3,3'-diindolylmethane (linear trimer or LT_1), 5,6,11,12,17,18-hexahydrocyclonona[1,2-b:4,5-b':7,8-b"]triindole (cyclic trimer or CT) and indolo[3,2-b]carbazole (ICZ) (Bjeldanes *et al.*, 1991; De Kruif *et al.*, 1991; Wortelboer *et al.*, 1992). It is through interactions of ACP's with the aryl hydrocarbon receptor (AHR) that I3C is believed to produce the majority of its effects, which have been proposed to include both chemoprevention and tumor promotion.

It was initially demonstrated that I3C and other indoles were chemoprotective when administered concurrently with the carcinogen or prior to initation (Wattenberg, 1977). Since then, I3C and DIM have been shown to be chemoprotective in a number of animal models and a variety of target organs (Morse *et al.*, 1990; Bailey *et al.*, 1991; Kojima *et al.*, 1994; Grubbs *et al.*, 1995;

Guo *et al.*, 1995; Oganesian *et al.*, 1997; Jin *et al.*, 1999; Chen *et al.*, 1998; Srivastava and Shukla, 1998). However, subsequent research demonstrated that chronic post-initiation exposure to I3C could potentially enhance carcinogenesis and result in significant promotion of liver tumors in rats and trout (Dashwood *et al.*, 1994; Kim *et al.*, 1994; Oganesian *et al.*, 1999; Stoner *et al.*, 2002).

Alteration of estrogen metabolism and the estrogen signal transduction pathway is one of the proposed mechanisms by which I3C could modulate carcinogenesis (Bradlow *et al.*, 1991; Baldwin and LeBlanc, 1992; Niwa *et al.*, 1994). Through the AHR, I3C has been shown to induce a number of phase I enzymes including cytochrome P450 (CYP). CYP1A1 and 1A2 catalyze the 2-hydroxylation of β-estradiol (E_2) and the shift towards higher levels of 2-hydroxy-E_2 may explain the chemoprotective effect exhibited by I3C or DIM against estrogen dependent cancers (Bradlow *et al.*, 1991; Jellinck *et al.*, 1993; Telang *et al.*, 1997; Wong *et al.*, 1997; Michnovicz, 1998). Other examples of antiestrogenic effects from I3C and I3C ACP's include the antiestrogenic properties of DIM in MCF-7 cells (Chen *et* al., 1998) and LT_1 functioning as an estrogen receptor antagonist in human breast cancer cell lines (Chang *et al.*, 1999). Conversely, I3C and I3C ACPs have been shown to exhibit some estrogenic activity as observed in the rainbow trout model (Shilling and Williams, 2000; Shilling *et al.*, 2001), and as an estrogen receptor agonist in human breast cancer cells (Riby *et al.*, 2000). An alternative mechanism for pro-estrogenic effects of I3C could be through induction

of CYP19 (aromatase) resulting in enhanced synthesis of estrogens (Sanderson *et al.*, 2001). Through estrogenic properties, I3C could enhance carcinogenesis by promoting estrogen dependent tumors.

In summary, it has been established that I3C and I3C-ACP's can have both estrogenic and anti-estrogenic characteristics and can act to both inhibit and promote carcinogenesis. It has also been demonstrated that the effects that estrogens have on carcinogenesis are species and tissue specific, as estrogens promote liver cancer in the rat (Yager and Yager, 1980; Wanless and Medline, 1982) while inhibiting chemically induced liver tumors in mice (Poole and Drinkwater, 1996). In the mouse, there is also a strain-dependent response to estrogens as modulators of liver cancer (Poole and Drinkwater, 1996). I3C shows similar characteristics as it is known to promote liver cancer in rats (Stoner *et al.*, 2002) and trout (Oganesian *et al.*, 1999) and inhibit DEN- induced hepatocarcinogenesis in C57 black mice (Oganesian *et al.*, 1997). I3C has also been shown to be estrogenic in trout (Shilling and Williams, 2000; Shilling *et al.*, 2001). This evidence indicates that I3C is most likely a modulator of liver cancer via estrogenic pathways. If this hypothesis is correct, then liver tumor formation will be inhibited by I3C feeding in a mouse strain that is estrogen responsive (C57BL/6J) and tumor formation should not be affected by I3C feeding in a mouse strain that is non-responsive with respect to liver cancer (C57BR/cdJ).

Materials and Methods

Chemicals

Diethylnitrosamine (DEN) and indole-3-carbinol were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals

C57BL/6J (black) and C57BR/cdJ (brown) mice were purchased from Jackson Laboratory (Bar Harbor, ME) as litters (10 litters per strain), each containing 5 male pups. All experimental procedures were conducted according to a protocol approved by the Oregon State University Institutional Animal Care and Use Committee.

Animal Treatment and Diets

At 15 days of age mice were injected i.p. with 5 mg/kg DEN in saline. DEN stock concentrations were prepared such that for each gram of body weight mice received 10 ul stock solution or saline for controls. The average body weight at day 15 was 5 grams. Nine days after injection mice were weaned and experimental diets started immediately.

Mice from each strain were divided into 4 treatment groups (Table 5.1). Groups 1 and 2 consisted of non-initiated (saline injected) animals, and received either control diet or diet containing 1500 ppm I3C, respectively. Groups 3 and 4 consisted of initiated (DEN injected) animals and also received either control diet or diet containing 1500 ppm I3C, respectively. For the brown mice, groups 1 and 2

consisted of 13 mice, and groups 3 and 4 consisted of 14 mice. For the black mice, groups 1 and 2 consisted of 12 mice, and groups 3 and 4 consisted of 17 mice. Prior to the completion of the study, two animals from brown group 1, one animal from brown group 2, one animal from brown group 3 and one animal from black group 2 died for reasons that were not related to treatment.

Diet preparation

AIN76A semipurified rat/mouse diet containing no synthetic antioxidants (butylated hydroxyanisole or butylated hydroxytoluene) was prepared using components from ICN (Costa Mesa, CA). I3C diets were prepared once per week, by adding I3C in powdered form to control diet, and storing at 4°C until used.

Animal Husbandry

Mice were kept at the Laboratory Animal Resource Center, Oregon State University, and maintained at 22°C and 40 to 60 % humidity on a 12 hour light/dark cycle. Lactating females with litters were housed in plastic cages with polyurethane covers. Pups remained with the mother until weaning (day 24). Once weaned, mice were housed one per cage, with diet and water available *ad libitum.* Mice were weighed weekly for the first three months and then bi-monthly thereafter.

Necropsy

After 7 months on experimental diets, mice were weighed and euthanized by CO_2 asphyxiation. Livers were removed, weighed and examined macroscopically for tumors. Tumors $\geq$ 0.5 mm in diameter were counted.

Statistics

Data were analyzed separately for brown and black mice. Body weight, liver somatic index and total tumor volume data were analyzed on the log transformed scale, while tumor multiplicity data were analyzed on the square root transformed scale. The transformations resulted in approximately normal and homogeneous errors. Linear mixed models were used that included, when necessary, random additive and non-additive (litter by treatment interaction) litter effects. Analyses were conducted using the Mixed Procedure in SAS version 8.2 (SAS Institute, Cary, NC (2001).

Results

Body Weight

At the conclusion of the study the average body weights for black and brown mice in the control groups were 37.5 and 53.9 grams, respectively (Table 5.1). I3C did not appear to have an effect on average body weight with the exception of causing a significant reduction to an average of 35.3 grams in black mice initiated with DEN (P=0.0032) (Figure 5.1). I3C did not contribute to any weight reduction in brown mice and despite a noticeable difference in the body weights of brown mice initiated with DEN, no significant difference was observed due to a large variation in response between litters (Figure 5.2).

TABLE 5.1. STUDY DESIGN AND DATA SUMMARY

Group	DEN (mg/kg)	I3C (ppm)	Final Body wt (g)	Liver wt (% of body wt)	Tumor Incidence	Total tumor volume per mouse
Brown 1	0	0	53.9±2.2	5.13±0.22	0/11	0
Brown 2	0	1500	51.6±2.1	5.46±0.30	0/12	0
Brown 3	5	0	43.2±0.7	10.90±0.89*	13/13	8485±1542
Brown 4	5	1500	42.4±1.3	11.76±1.16*	14/14	15375±3052
Black 1	0	0	37.5±1.2	5.00±0.41	0/12	0
Black 2	0	1500	37.0±0.7	5.60±0.23	0/11	0
Black 3	5	0	39.5±1.1	5.78±0.16	14/17	39.6±9
Black 4	5	1500	35.3±1.0*	5.72±0.20	14/17	824±551 (12.1±4.2)[1]*

All data given are means ± standard errors.
* = significantly different from corresponding control group, $P < 0.05$
[1] = mean and standard error with exclusion of outliers.

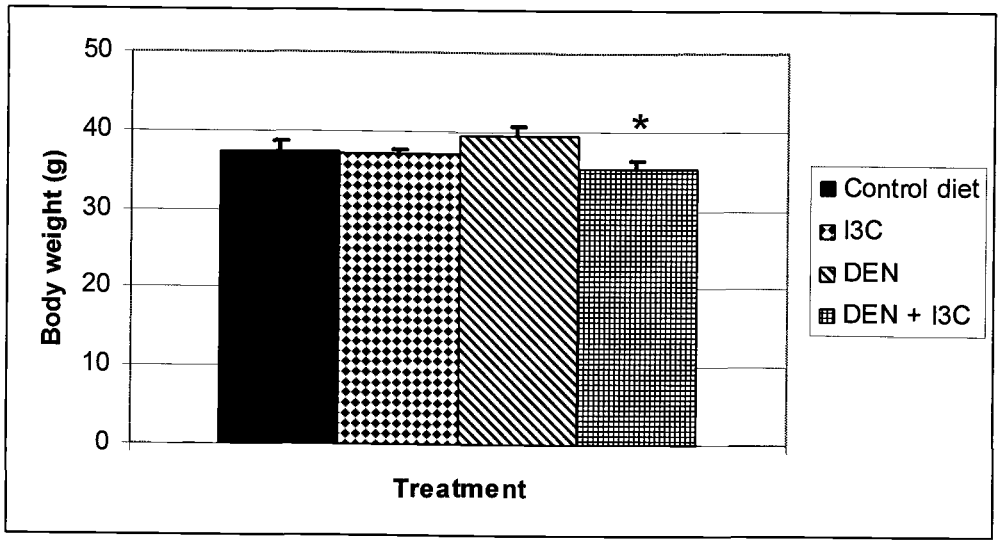

Figure 5.1: Body weights in black mice given an i.p. injection of either saline or 5 mg/kg DEN and then fed control diet or diet containing 1500 ppm I3C for 7 months. * = significantly different from corresponding control group, $P < 0.05$.

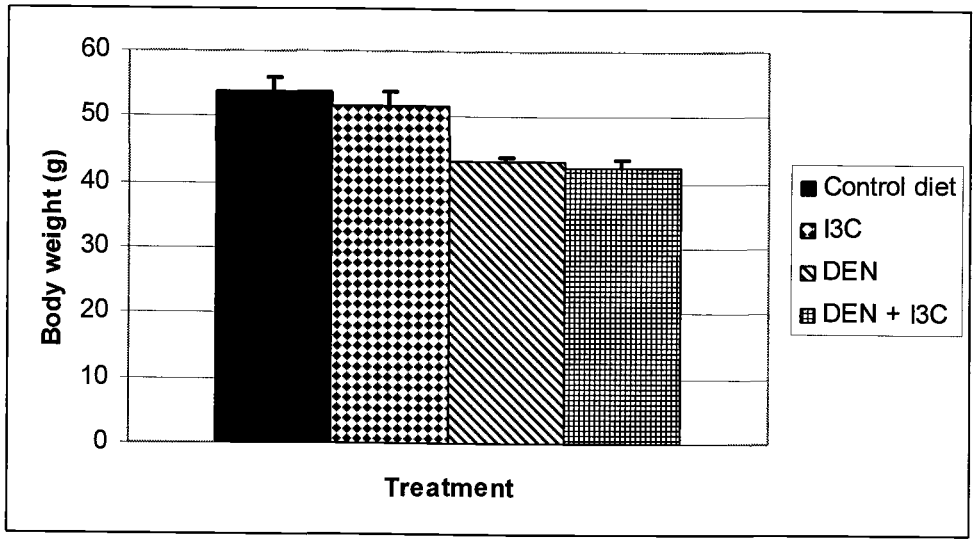

Figure 5.2: Body weights in brown mice given an i.p. injection of either saline or 5 mg/kg DEN and then fed control diet or diet containing 1500 ppm I3C for 7 months. Reductions in DEN initiated mice were not significant due to litter effects.

Liver Somatic Index

The liver somatic index of black mice was unaffected by I3C or DEN exposure in this study despite the presence of tumors in DEN initiated mice (Figure 5.3). Due to the extent of tumors observed in the brown mice exposed to DEN, and despite the previously mentioned effects that litter had on body weight, the liver somatic index in initiated mice was significantly higher (P=0.002) than observed in the corresponding control groups (Figure 5.4).

Tumor Incidence

Tumor incidence was not affected by chronic post-initiation exposure to I3C in either strain of mice (Table 5.1). In brown mice initiated with DEN, tumor incidence was found to be 100% in mice fed control diet (13 of 13) or diet containing 1500 ppm I3C (14 of 14). The groups of black mice that were initiated with DEN and either fed control diet or diet containing 1500 ppm I3C both had an 82.4 % incidence (14 of 17). No tumors were observed in non-initiated mice of either strain.

Tumor Volume

The total tumor volume for each mouse was calculated from the diameter measurement of each tumor by assuming the tumors were spherical (Table 5.1). In brown mice, feeding I3C enhanced tumor volume 1.8-fold but this did not reach statistical significance (p=0.06, simple t test). If one takes into account litter effects, there is little evidence of an effect of I3C on tumor volume in brown mice.

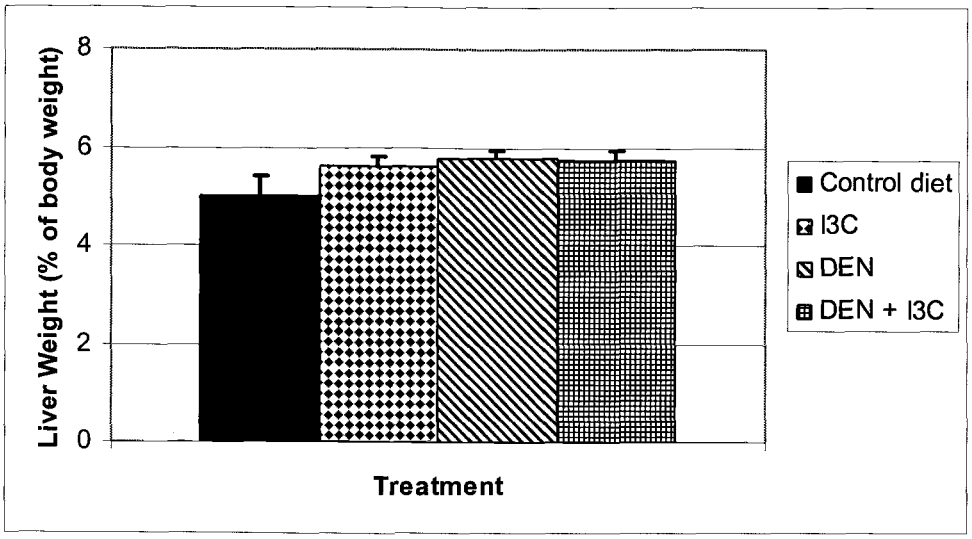

Figure 5.3: Liver somatic index in black mice.

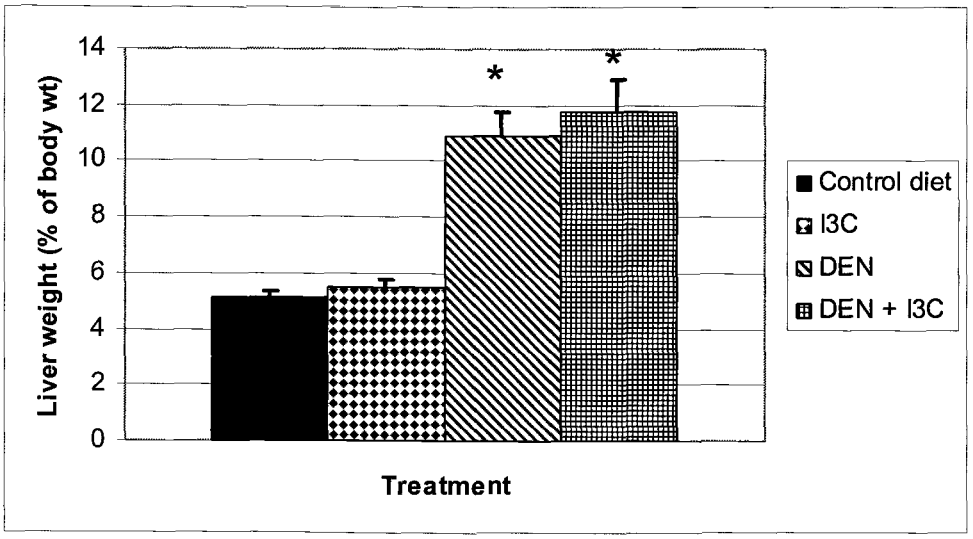

Figure 5.4: Liver somatic index in brown mice. * = significantly different from corresponding control group, $P < 0.05$.

In the black mice no litter effects were observed. If one considers all mice, there is an apparent 20-fold enhancement of tumor volume by the I3C diet. If the two mice with massive tumor volumes (50-60x next highest value) are considered outliers, I3C treatment results in an almost 3-fold reduction in tumor volume ($p<0.05$).

Tumor Multiplicity

Figures 5.5 and 5.6 display tumor multiplicity data for black and brown mice, respectively. I3C did not have a significant effect on tumor multiplicity in brown mice regardless of litter effects. If all black mice are considered, the small reduction in average tumor number, from 7.1 to 4.7, observed in mice fed I3C is also insignificant. As observed for the tumor volume data, if the two mice with extreme tumor burden are considered outliers, I3C treatment results in an almost 3 fold reduction in tumor multiplicity to 2.7 tumors per mouse ($p<0.05$). Brown mice were an order of magnitude more sensitive to DEN and had on average 90.2 and 82.8 tumors for the control and I3C treated mice, respectively.

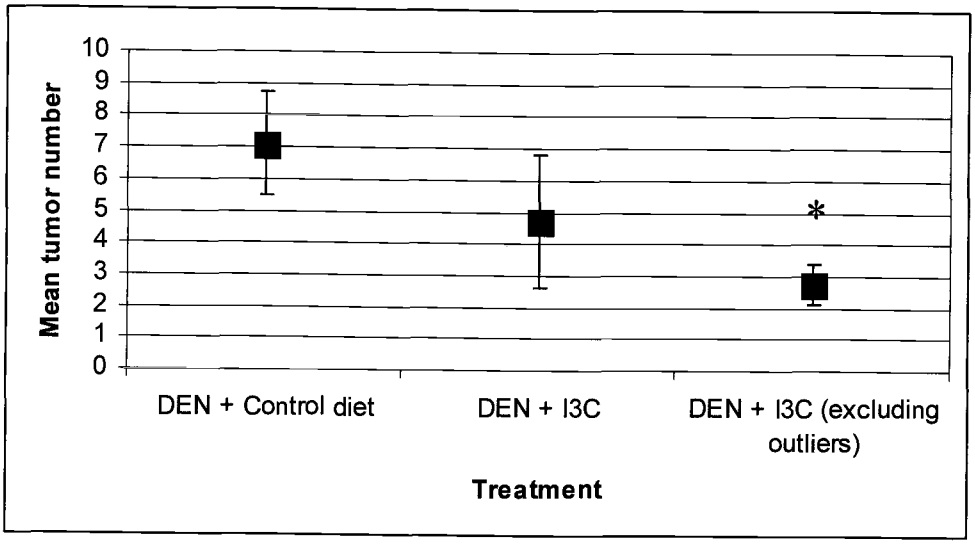

Figure 5.5: Tumor multiplicity in black mice initiated with DEN and fed either control diet or diet containing 1500 ppm I3C. No tumors were observed in non-initiated mice. * = $p<0.05$

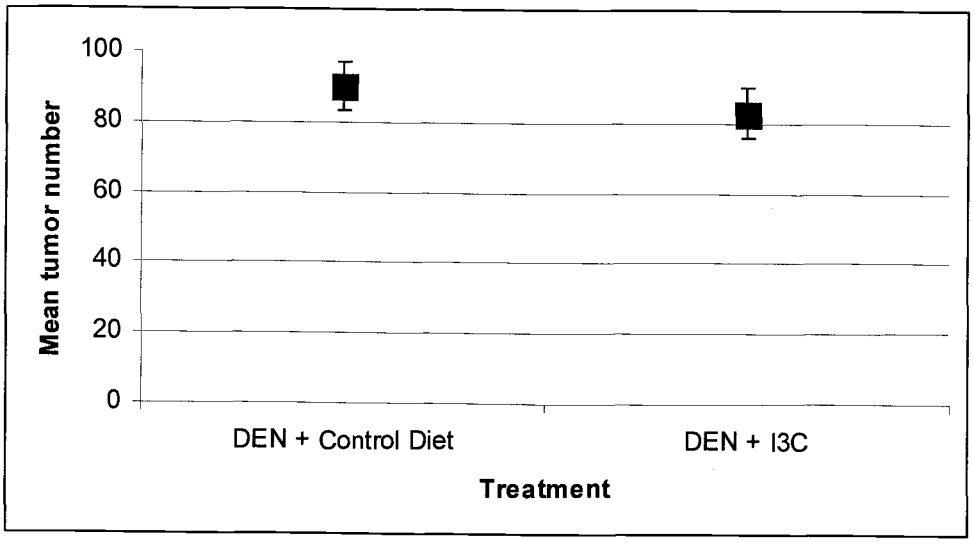

Figure 5.6: Tumor multiplicity in brown mice initiated with DEN and fed either control diet or diet containing 1500 ppm I3C. No tumors were observed in non-initiated mice.

Discussion

The purpose of this study was to investigate the mechanism by which chronic dietary post-initiation exposure to I3C inhibits hepatocarcinogenesis in mice (Oganesian *et al.*, 1997) while promoting liver tumors in rats (Stoner *et al.*, 2002) and trout (Dashwood *et al.*, 1994, Oganesian *et al.*, 1999). Interestingly, estrogens promote liver cancer in rat (Yager and Yager, 1980; Wanless and Medline, 1982), and trout (Nunez *et al.*, 1989) but function as inhibitors in most strains of mice (Poole and Drinkwater, 1996). This finding, along with evidence that I3C is estrogenic in trout (Shilling and Williams, 2000; Shilling *et al.*, 2001) indicates that I3C maybe modulating liver tumors via an estrogenic mechanism.

Two different strains of mice were utilized in this study in an attempt to clarify this issue. C57BL/6J (black) mice were selected as an estrogen responsive strain in which suppression of liver tumor development by estrogens is observed. C57BR/cdJ (brown) mice provided a non-responsive mouse model in which estrogens do not influence hepatocarcinogenesis (Poole and Drinkwater, 1996). If I3C were modulating hepatocarcinogenesis by estrogenic mechanisms then the inhibition of liver tumors previously observed in black mice exposed to I3C would not be observed in brown mice exposed to the same treatment.

The large discrepancy in sensitivity to DEN between the black and brown mice observed in this study was not expected. Female brown mice are known to be more susceptible to hepatocarcinogenesis because they are highly insensitive to the

suppressing effects of ovarian hormones such as estrogen but the male brown mice used in this study are intermediate in sensitivity among inbred mouse strains (Poole and Drinkwater, 1996).

In this study, I3C treatment did not have a significant influence on tumor modulation in brown mice, despite resulting in a 1.8-fold enhancement in total tumor volume per mouse. The 3-fold reduction in tumor multiplicity and average total tumor volume observed in black mice is consistent with a chemopreventive effect of I3C previously observed in this same C57BL/6J infant mouse model with DEN initiation (Oganesian *et al.*, 1997). The slight but insignificant increase in tumor volume in brown mice along with the reduction in tumor multiplicity and volume in black mice supports the hypothesis of I3C acting as an estrogen. This could be significant for determining the suitability of I3C supplementation in humans for preventing estrogen-related disease without increasing the risk of hepatocarcinogenesis.

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CHAPTER 6

Summary and Conclusions

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Summary and Conclusions

Indole-3-carbinol (I3C) and 3,3'-diindolylmethane are rapidly becoming two of the top selling phytochemical supplements and are under investigation as potential chemopreventive agents, in spite of limited information on the effects of chronic exposure. Previous studies have demonstrated that the chemopreventive potential of I3C and DIM in animal studies is dependent on species, strain, tissue and timing of treatment relative to carcinogen exposure. The majority of biological effects from I3C are the result of DIM and other I3C-ACPs binding to the aryl hydrocarbon receptor and the subsequent induction of phase I and phase II enzymes. The levels of these enzymes can readily influence carcinogenesis by blocking the action of carcinogens or increasing their rate of bioactivation. Modulation of enzyme levels can also result in altered metabolism of compounds that could affect efficacy and toxicity of pharmaceuticals and xenobiotics. The current work focused on the effects of chronic dietary I3C and DIM exposures on health, drug metabolism and carcinogenesis in rodent models.

The only sign of toxicity from either compound was a reduction in body weight in Fischer 344 rats treated with 2500 ppm dietary I3C for 12 months. Toxicity was not confirmed by blood chemistry or histopathological analysis and, furthermore, no toxicity was observed in Sprague-Dawley rats after a comparable exposure. In fact, a general decrease in serum enzyme levels in male rats of both strains may point towards a potential protective effect from age related tissue damage.

Previous studies have demonstrated that both I3C and DIM are capable of modulating the levels of monooxygenase enzymes and therefore have the potential to alter *in vivo* drug metabolism. This was confirmed by the current work in which chronic exposures to I3C and DIM resulted in significant induction of total hepatic cytochrome P450 (CYP). CYP1A and CYP3A were induced up to 80- and 30- fold, respectively, depending on the strain of rat and length of exposure. DIM was a less efficacious inducer of CYP and also caused less modulation to drug metabolism in liver slice incubations.

As previously observed, chronic post-initiation exposure to I3C resulted in an apparent chemoprotective effect against DEN initiated hepatocarcinogenesis in black mice. Alternatively, tumor size, incidence, and multiplicity were not significantly altered by I3C exposure in brown mice. These results suggest that I3C modulates hepatocarcinogenesis in mice via an estrogenic mechanism.

These studies demonstrate that chronic I3C and DIM exposures do not produce appreciable direct toxicity in rodents, but adverse effects related to exposure could occur through indirect mechanisms. Chronic post-initiation exposure did not result in tumor promotion in this study but the observed induction of CYP 1A has been linked to increased cancer risk through greater carcinogen bioactivation. The alterations in monooxygenase levels and the associated effects on metabolism confirm our concern that chronic I3C and DIM exposure may result in significant effects on drug and/or xenobiotic metabolism.

The results of the current work, along with previous studies demonstrating that I3C causes toxicity in some models and can promote tumors, brings into question the benefit versus risk that comes from I3C and DIM supplementation. If results from these studies in rodents, following chronic exposure of I3C and DIM, are indicative of the human response then direct toxicity from long-term supplementation in humans should not be the foremost concern. The major implications for human health would be possible alterations in drug and/or xenobiotic metabolism that could lead to drug interactions or induction of carcinogenesis. These risks, in addition to the previously mentioned work that demonstrated the lowest dose of I3C to inhibit mammary tumors in the rat was potent enough to induce lesions in the liver, indicate that the effect of chronic supplementation with I3C and DIM in humans maybe too uncertain for preventative use. Conversely, if trials of I3C and DIM as treatments for estrogen dependent disease like RRP and CIN are successful, the benefit of long-term exposure would in this case overcome the risk. Additional studies should be completed to further determine the effects of chronic exposure to I3C and DIM in humans.

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